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The Effects of Punicalagin and Tannic Acid on Caenorhabditis elegans Models of Alzheimer's Disease

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The Effects of Punicalagin and Tannic Acid on *Caenorhabditis elegans* Models of Alzheimer's Disease

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Table of Contents

Table of Figures

Abstract

Alzheimer's disease is the fifth leading cause of death in the United States and the rate of diagnosis is increasing steadily. It is imperative to understand the pathology of the disease and its underlying mechanisms in order to develop potential therapies. This neurodegenerative disease involves neuronal death and brain atrophy associated with the presence of amyloid-β plaques. Alzheimer's disease leads to impaired memory and cognitive skills, as well as the disruption of everyday tasks, such as movement and speech. Currently there is no cure for Alzheimer's disease; there are only therapies intended to manage symptoms. Our study focused on *Caenorhabditis elegans* models of Alzheimer's disease and the ability of natural extracts to prevent the onset of symptoms caused by the disease. *C. elegans* is often used in the scientific community as a model organism due to the fact that its entire genome is sequenced and mapped, its neuronal connections are known – making research easier and more effective. We studied the effects of two polyphenols found in plants, punicalagin and tannic acid, as they have been shown to have antioxidant and anti-inflammatory properties, implicating neuroprotective effects. In this study, *C. elegans* behavioral assays were used to study the effects of punicalagin and tannic acid in combatting the effects of the amyloid-β peptide. These assays tested the chemosensation and the muscular deficits as Alzheimer's disease can cause olfactory defects and paralysis in patients. Our results suggested that punicalagin reduces the neuronal deficits caused by the accumulation of the amyloid-β peptide, while tannic acid was not shown to have a rescuing effect.

Acknowledgements

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1 Introduction

Alzheimer's disease is the fifth leading cause of death in the United States, and is the only of the top ten deadly diseases that cannot be prevented, treated, or cured (Centers for Disease Control). This devastating disease has led to more deaths than both breast and prostate cancers combined. It is projected that the number of new Alzheimer's disease diagnoses will triple by mid-century (Gaugler, James and Johnson). Although the complete pathology of the disease is not fully understood, the most supported theory as to how the disease progresses is outlined in the Amyloid Cascade Hypothesis, depicted in Figure 4 on page 15 (Hardy and Selkoe). The hypothesis states that through the accumulation of an isoform of amyloid-β protein that consists of 42 amino acids, extracellular plaques form, leading to neuronal death and neurodegeneration. This in turn causes the cognitive and physical symptoms exhibited by Alzheimer's patients (Games, Adams and Alessandrini)

Current therapeutic research on an Alzheimer's cure and symptom alleviation is hindered by obstacles such as the selection of the right target of interest in the disease pathway, or the delivery of molecules across the blood brain barrier (Laterra, Keep and Betz). Examples of possible targets include butyrylcholinesterase, β- and γ-secretases, and the α7 nicotinic receptor, all of which have been implicated as possible causative agents of the disease (Greig, Utsuki and Yu) (Kem) (Scarpini, Schelterns and Feldman). However, many of these targets also have other functions in the body, making it difficult to determine how they can be safely manipulated (Greig, Utsuki and Yu) (Kem) (Scarpini, Schelterns and Feldman). Therefore, study of potential therapies for challenging diseases such as Alzheimer's disease is often done on simpler organisms, which are commonly called model systems. These systems are organisms or cells that can be experimentally manipulated and used to understand biological functions. This data can be extrapolated to other organisms including humans, due to genetic conservation between species.

There are many organisms used as models for Alzheimer's disease including mice, rhesus monkeys, and *Caenorhabditis elegans* (Van Dam and De Deyn). Commonly known as the roundworm, *C. elegans* is a nematode that has a nervous system which functions similarly to the human nervous system, but is simple enough to be studied in depth. Their body consists of 959 cells, of which 302 are neurons and 81 are muscle cells. The network of connections between these neurons is completely mapped, allowing for a deeper understanding on the worm's nervous

system, including genes, pathways, receptors, and molecules necessary for normal function. *C. elegans* can easily be genetically manipulated with very little cost. This allows for the insertion or deletion of genes of interest, which is very useful in the study of genetic diseases. Coupled with the worms' ease of propagation and high rate of reproduction, *C. elegans* is a great model organism to study neurodegenerative diseases such as Alzheimer's disease (Kaletta and Hengartner).

We used *C. elegans* strains that were genetically modified to express the human amyloid-β_{42} ($A\beta_{42}$) transgene either pan-neuronally or pan-muscularly, in order to assess the behavioral effects of the peptide. Using three behavioral assays to evaluate either the chemosensory or muscular phenotypes of the worms, we characterized the behavioral defects caused by the $A\beta_{42}$ transgene. We subsequently investigated the effects of treating the worms with plant-derived extracts, and the degree of alleviation of observed behavioral defects.

A group of compounds that has been implicated as potential drug therapies for Alzheimer's disease is that of plant-derived extracts. Researchers have been focusing on natural extracts because historically these extracts have led to the discovery of effective drugs, such as morphine, and they are both cheap and readily available (Bastianetto and Quirion). Two compounds that have previously been found to have rescuing effects against the symptoms of Alzheimer's disease are punicalagin and tannic acid. Punicalagin and tannic acid are polyphenols with antioxidant and anti-inflammatory properties, found in pomegranates and red wine, respectively (Olajide, Kumar and Velagapudi) (Ono, Hasegawa and Naiki). At the start of this study, there was no published data regarding these extracts being tested in *C. elegans* models of Alzheimer's disease. Therefore, for our project, we investigated the effects of these natural compounds on *C. elegans* models of Alzheimer's disease.

Our results indicate that punicalagin prevented the onset of symptoms caused by $A\beta_{42}$ that was expressed pan-neuronally, but not those caused by pan-muscular expression. In contrast, tannic acid did not have an effect on neither the chemosensory nor muscular defects that were caused by the peptide, and possibly even exacerbated the effects of $A\beta_{42}$. It is possible that the concentration of tannic acid used in this project was too high, resulting in a toxic effect. From the conclusions gathered by this project, there are numerous directions that future research can take. Studies could investigate the neuroprotective mechanism of punicalagin, or assess the possibility of punicalagin as a retroactive treatment for Alzheimer's symptoms.

2 Background

2.1 The Human Nervous System

The human nervous system transmits stimuli from sensory receptors throughout the body to the brain and spinal cord, before sending impulses back to the body in order to elicit a response (Matthews). The building blocks of the nervous system are small cells called neurons, which can be electrically excited. This allows neurons to transmit information through electrochemical signaling. This signaling is carried out by neurotransmitters: chemical messengers which relay, modify, and regulate information between neurons (Boeree). Neurons are incapable of dividing and cannot be regenerated: if a neuron dies, the functionality of that neuron is permanently lost.

2.1.1 The Central and Peripheral Nervous System

The nervous system is functionally distinguished into two separate classifications: the central nervous system (CNS) and the peripheral nervous system (PNS) (Mangels). The CNS consists of the brain and spinal cord. The brain acts as the information processor and coordinates both conscious and unconscious body functions, while the spinal cord serves as the connection between the brain and the PNS (Timiras). The PNS consists of all other nerve cells throughout the body. Nerves of the PNS have either sensory or motor functions. Sensory neurons recognize changes in the environment by communicating with sensory receptors throughout the body, then relay information regarding these changes to the CNS. After processing, the CNS passes the information to the motor neurons that regulate muscle movement (Mangels).

The motor neurons in the PNS can be further separated into two divisions: somatic (voluntary) and autonomic (involuntary). The somatic motor neurons are responsible for movement of skeletal muscles and function of sensory receptors under the skin (Swenson). The autonomic motor neurons regulate smooth muscles that cannot be controlled consciously, such as the heart and stomach (Mangels).

2.1.2 The Function of a Neuron

Neurons have four main structural components: the cell body, the dendrite, the axon, and the axon terminal. These structures can be seen in Figure 1. The cell body contains the nucleus, and is also a main location of protein synthesis. The rest of the neuron's structure is mainly involved with electrical signaling. First, a neuron receives chemical signals from other neurons at the dendrites. These signals are converted into electrical impulses and are then transmitted to axons. At the axon terminal, these impulses are converted back to chemical signals in the form of

neurotransmitters and released into the synapse, where they are received by the next neuron. The axon terminal is also involved in the reuptake of some of these neurotransmitters (Lodish, Berk and Zipursky).

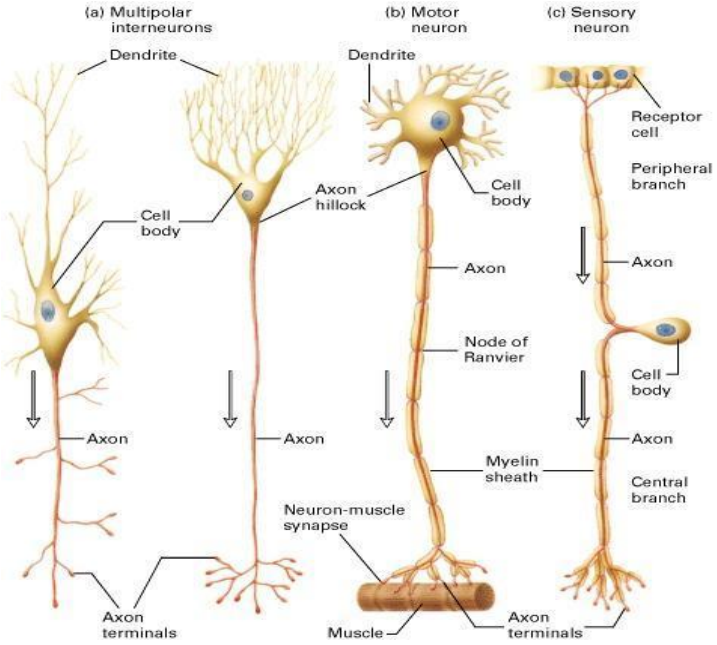

Figure 1. *Neuron Structures. Electrical signals are received by dendrites, passed through the cell body and to the axons. At the end of the axons chemical signals are released to the next neuron through the axon terminals (Lodish, Berk and Zipursky).*

2.1.3 Neurodegeneration and Neurodegenerative Diseases

Neurodegeneration is defined as the gradual loss of the structure or function of neurons, typically resulting in a deterioration of brain function (Przedborski, Vila and Jackson-Lewis). This may happen naturally due to aging, or as a result of various diseases. Several factors influence the potential for neurodegeneration including genetic mutations, toxic environmental factors, or a combination of the two (Przedborski, Vila and Jackson-Lewis). Currently, there is no drug for either treatment or prevention of these diseases.

Some of the major neurodegenerative diseases include amyotrophic lateral sclerosis (ALS), Huntington's disease, Parkinson's disease, and Alzheimer's disease. At the cellular level, each of these diseases has been found to involve an abnormal buildup of proteins in the central nervous system, which leads to neurodegeneration and eventual neuronal death (Peden and Ironside).

ALS is a neurodegenerative disease that manifests as damage within the neurons located in a patient's spinal cord. This damage occurs in response to the aggregation of ubiquitinated proteins (Blokhuis, Groen and Koppers). With the progressive degeneration of motor neurons in

this area, the brain loses its connection to the neurons that initiate and regulate muscle movement. The loss of muscle movement leaves patients unable to speak, eat, or breathe, which ultimately leads to death (ALS Association).

On the other hand, Huntington's disease affects the brain itself, with some areas being more susceptible to damage than others. In this disease a protein called huntingtin is prone to misfolding and aggregation within the brain (Arrasate and Finkbeiner). One of the areas more susceptible to this aggregation is the basal ganglia, which is composed of nerve cell clusters called nuclei. These cells play an important role in both movement control and behavior, which explains why there is deterioration of these actions in a Huntington's disease patient. Huntington's disease is a genetically inherited, early onset disease, with symptoms appearing as early as 30 years of age (Huntington's Disease Society of America).

Parkinson's disease typically occurs later in life, and is characterized by clumps of proteins called Lewy Bodies, as well as neuronal death, primarily in an area of the brain called the substantia nigra. This neuronal death leads to decreased production of dopamine, which is involved in relaying information to the primary motor cortex and subsequently controlling voluntary motion and coordination. The decrease in dopamine in the brain causes a patient with Parkinson's disease to progressively lose the ability to control body movements. Except in rare cases of familial Parkinson's disease, the exact cause and mechanism of the protein buildup and subsequent disease progression remains unclear (Parkinson's Disease Foundation).

Another neurodegenerative disease that typically occurs later in life is Alzheimer's disease. Alzheimer's disease is the most common cause of dementia, and causes memory loss and decreased cognitive ability. This disease is characterized by the accumulation of amyloid-β and tau proteins within the brain that leads to eventual neuronal death. This disease inhibits an individual from performing everyday tasks, which eventually prevents him or her from living independently. Ultimately, Alzheimer's disease leads to death (University of California, San Francisco).

2.2 Alzheimer's disease

The major focus of this research project was Alzheimer's disease. Attempts were made to alleviate the symptoms displayed by *C. elegans* genetically engineered to express $A\beta_{42}$. As such, a deeper understanding of the disease itself is necessary to put this research project into context.

The following section will further discuss the current state of Alzheimer's disease understanding, prevalence, and drug development.

2.2.1 Discovery, Prevalence, and Symptoms

First described by Dr. Alois Alzheimer in 1906 (Alzheimer's Disease International), Alzheimer's disease is the most common form of dementia. It currently affects approximately 5.3 million Americans (Gaugler, James and Johnson), and is in the top five leading causes of death in the United States (Centers for Disease Control). The neuronal damage that occurs as a result of Alzheimer's disease impairs memory, cognitive skills, and performance of vital bodily functions, ultimately leading to death (Gaugler, James and Johnson).

An estimated one in nine Americans aged 65 or older have Alzheimer's disease. This frequency jumps to one in three for those aged 85 and older. However, this is likely an underestimate, as Alzheimer's disease is underdiagnosed. Many believe that the disease begins manifesting in an individual prior to the appearance of symptoms, an idea that, once validated, will likely increase the number of diagnoses (Gaugler, James and Johnson).

Additionally, these statistics only take into account individuals who have died as a direct result of Alzheimer's disease, as described on their death certificate. This number underestimates the number of deaths resulting from the disease, as many acute conditions that lead to death, such as pneumonia, are caused by the complications associated with Alzheimer's disease (Gaugler, James and Johnson). These complications include immobility and malnutrition, both of which leave a patient more susceptible to other potentially deadly conditions. This blurred line between dying with the disease and dying from the disease implies that there are more deaths with Alzheimer's disease as the underlying cause than current data implies. The Alzheimer's Association explains that "[r]egardless of the cause of death, among people age 70, 61 percent of those with Alzheimer's are expected to die before age 80 compared with 30 percent of people without Alzheimer's."

While the current diagnosis numbers are staggering, predictions for future incidence of Alzheimer's disease diagnoses are significantly worse. As of 2011, the generation referred to as the "baby boomers" began to reach age 65.[1] This generation has benefitted from modern medicine

[1] The term "baby boomers" alludes to those born during the years 1946-1964. These were the years following World War II, when there was a sharp increase in the birth rate in the United States (L'Allier and Kolosh).

and public health advancements, leading to longer life expectancies than for their predecessors (L'Allier and Kolosh). These factors lead experts to project an almost threefold increase in the number of Americans aged 65 or older with Alzheimer's disease by 2050 (Gaugler, James and Johnson). Figure 2 displays the predicted numbers of Americans with Alzheimer's disease from 2010 through 2050. Assuming these predictions are accurate, millions more Americans will die prematurely as a result of Alzheimer's disease in the coming years.

As Alzheimer's disease is a slow, progressive form of dementia, the majority of patients live many years with the disease. The average survival time for an individual diagnosed with Alzheimer's disease is 4-8 years, but some patients live significantly longer (Gaugler, James and Johnson). An average of 40% of the time living with Alzheimer's disease is spent in the most

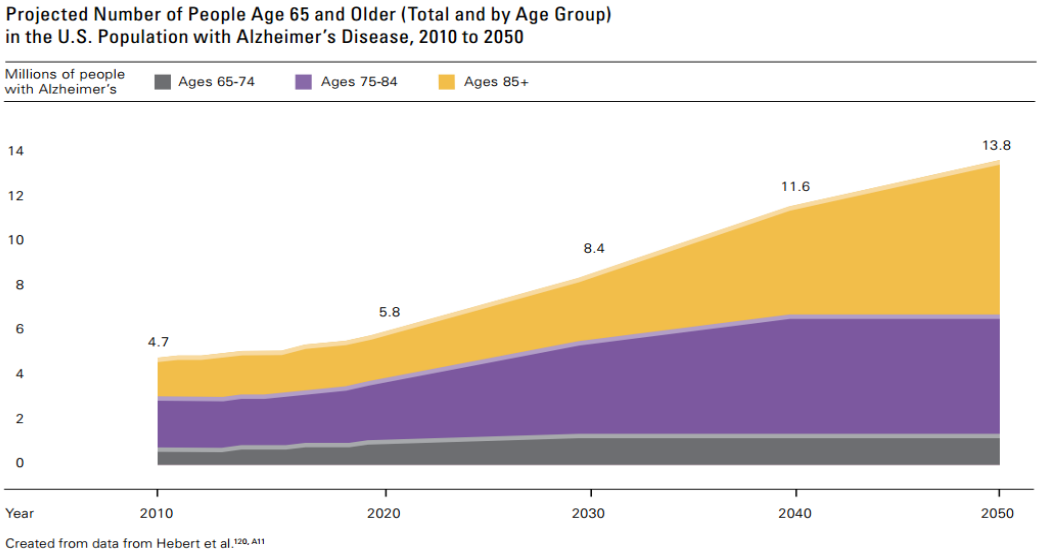

***Figure 2**. Projected Number of Americans Age 65+ with Alzheimer's Disease, 2010-2050 (Gaugler, James and Johnson).*

dependent stage of the disease (Gaugler, James and Johnson). In this final stage, patients are unable to carry out daily tasks or care for themselves, and typically either live with family or are admitted to a nursing home.

When family members of Alzheimer's patients take on caregiving duties, the monetary, time, and emotional costs are enormous. It is estimated that these informal caregivers spent almost 18 billion hours assisting individuals with Alzheimer's disease in 2014 alone, with this care being valued at about $218 billion (Gaugler, James and Johnson). Out-of-pocket expenses related to Alzheimer's disease for patients 65 and older tend to be more than $10,000 per year. These expenses double for patients living in nursing homes or assisted living. Additionally, individuals

with Alzheimer's disease accrue "three times as many hospital stays per year as other older people" (Gaugler, James and Johnson), leading to even more medical expenses. This monetary burden is not restricted to the individual families paying for treatment and care. Medicare and Medicaid are government programs that cover the majority of the expenses for low-income individuals. As a large number of Alzheimer's disease patients rely on these services and require several years of treatment and care, the disease results in very high costs for these programs, translating to higher costs for taxpayers (Gaugler, James and Johnson).

A diagnosis of Alzheimer's disease adds emotional stress on both the patient and his or her family. There are many difficulties attributed to Alzheimer's disease, such as dealing with the patient's loss of judgment, understanding, and ability to communicate. Behavioral changes can also be difficult for a family member or friend to handle. Feelings of depression, guilt, and a lack of control are reported in the majority of caregivers (Gaugler, James and Johnson). Alzheimer's disease affects the patient as well as their family and friends.

2.2.2 Disease Pathology

The progressive cognitive decline that occurs in Alzheimer's disease has a devastating effect on all aspects of the patient's life. The pathology of this decline is not yet fully understood, but autopsies of Alzheimer's patients have led to the conclusion that the disease causes extensive neuronal death and brain atrophy. In fact, autopsy is currently the only way to definitively diagnose any individual with Alzheimer's disease (Perl).

One component of this post-mortem Alzheimer's diagnosis is the presence of neurofibrillary tangles (Perl). Neurofibrillary tangles are insoluble fibers found inside of neurons. They are mostly made up of tau proteins which are associated with microtubules (Paudel). Hyperphosphorylation of tau proteins within these neurofibrillary tangles is one of the hallmarks of Alzheimer's disease, and the amount of tangles has been shown to correlate with both the length and degree of severity of the disease (Perl).

Amyloid plaques are the other primary component of Alzheimer's disease. Similar to neurofibrillary tangles, amyloid plaques are accumulations of insoluble protein fragments. These consist of amyloid-β (Aβ) peptides that stick together to form the plaques (Games, Adams and Alessandrini). Unlike neurofibrillary tangles, these plaques are found outside of the neuronal body (Paudel). Both amyloid plaques and neurofibrillary tangles are shown in Figure 3.

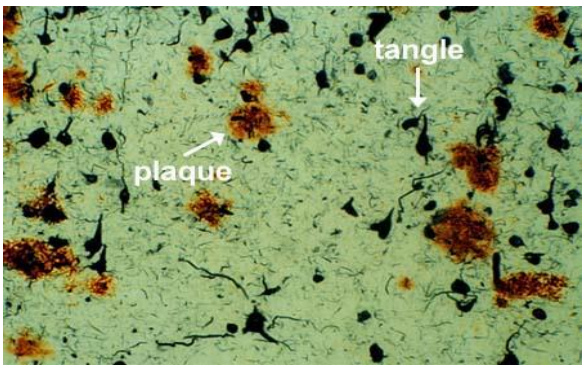

Figure 3. *Depiction of amyloid plaques and neurofibrillary tangles in an Alzheimer's disease patient (LaDu)*

Though the exact pathology of Alzheimer's disease is not known, the most widely-accepted hypothesis is that the accumulation of amyloid-β peptide in the brain leads to disease development (Hardy and Selkoe). This hypothesis, known as the Amyloid Cascade Hypothesis, is summarized in Figure 4. Central to this hypothesis is Amyloid Precursor Protein (APP), which is cut by γ-secretase and BACEs (β-APP cleaving enzymes) to form amyloid-β (Karran, Mercken and De Strooper). The cut sites of γ-secretase vary, forming amyloid-β fragments that range from 39-43 amino acids long. The most commonly found isoforms are either 40 or 42 amino acids long, denoted as $A\beta_{40}$ and $A\beta_{42}$, respectively (Karran, Mercken and De Strooper). The amyloid plaques found in Alzheimer's disease are made up of primarily $A\beta_{42}$. Higher amounts of $A\beta_{42}$ have been shown to correlate with higher levels of neuronal death (Gu, Guo and Zhefeng).

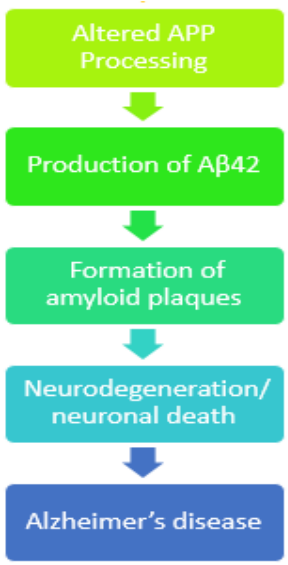

Figure 4. *Summary of the Amyloid Cascade Hypothesis*

While $A\beta_{42}$ leads to plaque formation, what causes the initiation of neurofibrillary tangle formation remains unknown. Current research suggests that the neurofibrillary tangles form after plaques, and may even be prompted by their formation (Hardy and Selkoe). This research has helped the Amyloid Cascade Hypothesis gain acceptance; however there are still some who argue

for a tau hypothesis, whereby tau aggregation is considered the causative agent for Alzheimer's disease (Maccioni, Farías and Morales).

Regardless of whether the disease is prompted by amyloid plaque or neurofibrillary tangle formation, the high rates of neuronal death in Alzheimer's disease lead to a loss of synaptic connections within the brain. Without the ability to receive and transmit signals, the surviving neurons become non-functional as well. Eventually the degradation becomes widespread and the brain shrinks, as shown in Figure 5. The neuronal degradation and shrinking of the brain cause the cognitive symptoms and eventual death of Alzheimer's disease patients (National Institute on Aging).

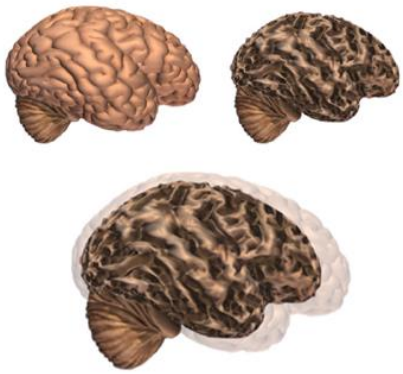

Figure 5. *Image showing a healthy brain (top left), an Alzheimer's disease brain (top right), and the two overlapped to directly show the size comparison (bottom) (Gaugler, James and Johnson).*

2.2.3 Current Treatments

Although Alzheimer's disease has been studied extensively for many years, there are many obstacles in the way of finding a cure. Uncertainty regarding the disease pathology has led to debate over what aspects of the disease should be targeted by therapeutics. A variety of clinical trials of therapies for prevention and treatment have been conducted, with limited success. Some of these therapies have included secretase inhibitors to prevent formation of amyloid-β and promote amyloid-β clearance, anti-inflammatory drugs, cholesterol lowering drugs, and neuroprotective compounds to prevent disease onset and progression (Hardy and Selkoe).

There are many obstacles to overcome when working with the brain. The blood brain barrier is the biggest obstacle in treating any disease of the brain. The blood brain barrier is the brain's specialized "security system", and acts to allow essential nutrients access to the brain while blocking potentially harmful chemicals. This barrier adds a layer of complexity to treating brain disorders, as it hinders drug delivery (Laterra, Keep and Betz). Potential therapeutics for

Alzheimer's disease must be engineered so that they are able to pass through the blood brain barrier.

Additionally, many of the molecules involved in Alzheimer's disease's suspected pathology are also involved in other processes in the body. A notable example of this is γ-secretase. Because γ-secretase is necessary to cut APP into the toxic $A\beta_{42}$, a potential therapeutic could be to inhibit γ-secretase in order to eliminate $A\beta_{42}$ aggregation. Unfortunately, γ-secretase is also important to Notch protein and other cell surface receptor signaling (Hardy and Selkoe). Notch proteins are essential in cell signaling and development throughout the body (Kopan and Ilagan). As such, inhibition of γ-secretase would likely have many off-target consequences.

2.3 Model Systems in Biology

Before any potential treatment for Alzheimer's disease can be tested in humans it must be tested on model systems. In this research project the model system used was the nematode *C. elegans*, however there are a multitude of options for model systems when studying biology.

Model systems are used to study all aspects of biology – including simple biological functions, behaviors, diseases, and drug discovery. These systems can be as small as single cells or as large as whole populations. The criteria which govern whether or not an organism would make a good model system includes its size, life cycle, rate of reproduction, ease of maintenance, and ability to be manipulated for experimental purposes. By accruing large amounts of data regarding a few model organisms, scientists are able to extrapolate and predict how changing conditions may affect other organisms in terms of health and behavior (Kunkel).

Some model systems have long life cycles and are not ideal for genetic manipulation, but can be used to study behavior and reproduction. Examples of these model organisms include the chicken (*Gallus gallus*) and the frog (*Xenopus laevis*), which both breed multiple times each year. These models are large and complex, and have more in common with humans than smaller model organisms (Twyman). Some large, complex organisms, such as mice, are used to test the safety and efficacy of drug treatments prior to beginning clinical trials in humans (Steinmetz and Spack).

Organisms with short life spans that reproduce in large numbers allow for the study of genetics over generations. Genetic crosses can be performed and mutations can be introduced through various molecular methods. This allows for the study of specific gene functions and interactions (Twyman). Examples of genetic model organisms include the baker's yeast *Saccharomyces cerevisiae*, the fruit fly *Drosophila melanogaster,* and the nematode *C. elegans*.

2.3.1 *C. elegans* as a Model Organism

C. elegans is a model organism used to study various topics in biology. Of the phylum Nematoda, *C. elegans* are non-parasitic, free-living roundworms, which live in the soil, as well as in decaying fruits and vegetables throughout the world. These worms undergo morphogenesis including larval stages (denoted L1 through L4) and an adult stage, all of which are visible under a microscope. This developmental cycle lasts about two to three days when maintained 20°C, with different temperatures leading to different growth rates (Edgley). An adult worm is shown in Figure 6.

Figure 6. *Adult C. elegans under 40X magnification*

Various factors make *C. elegans* an ideal model organism for our research. *C. elegans* is easy to maintain in the lab – the worms can be grown on petri dishes containing Nematode Growth Medium (NGM) agar seeded with a bacterial food source, most commonly *Escherichia coli*. The ease and low cost of maintenance make *C. elegans* an effective tool for gathering large amounts of data. Another important factor is that the developmental cycle is both short as well as consistent between animals. This allows for experiments to be run quickly and at specific developmental stages (WormAtlas). Additionally, much is already known about *C. elegans*. In 1998 the entire genome of *C. elegans* was mapped, marking the first time an animal genome was sequenced (The C. elegans Sequencing Consortium). This provided a wealth of important genetic information that has aided in the understanding of gene function and provided the ability to perform genetic manipulations. These manipulations are easily maintained in a strain, as more than 99% of *C. elegans* are self-fertilizing hermaphrodites that produce up to 1,000 genetically identical progeny (Corsi). Years of research have led to the development of well characterized behavioral assays designed to test everything from simple behaviors such as foraging and sensory responses, to complex behaviors such as male mating, and learning and memory.

2.3.2 *C. elegans* and Alzheimer's disease

C. elegans is an effective model organism for the study of diseases. While it shares many characteristics with humans, including neuronal structure and communication, it is simple enough at the molecular and cellular level to be studied in depth (Hart and Chao). This allows for important insights into disease mechanisms and pathways that cannot be easily elucidated from more advanced organisms. As previously mentioned, about one third of the somatic cells in *C. elegans*

are neurons (Edgley). The neuronal network has been completely mapped, making *C. elegans* useful tools for studying diseases of the nervous system (Riddle).

$A\beta_{42}$ is a necessary component of Alzheimer's disease pathology, and as such any model used to test the disease must include this protein. Unfortunately, *C. elegans* lack β-secretase, so $A\beta_{42}$ cannot be produced from APP (Wolozin, Gabel and Ferree). In order to harness the power of the *C. elegans* model organism as a tool to study Alzheimer's disease, strains were created by the Link lab at the University of Colorado to include the human $A\beta_{42}$ transgene. This allowed for high throughput drug screening, similar to what is done with cultured neurons but with the added benefit of the organismal complexity of a living animal (Lublin and Link). The transgene was designed to express $A\beta_{42}$ in either all of the neuronal or all of the muscle cells of the animal. The pan-neuronal transgene is expressed with a temperature sensitive promoter, so that $A\beta_{42}$ is expressed when worms are exposed to temperatures above 20°C. Because of this, the worms must be propagated at 16°C. Two *C. elegans* strains expressing $A\beta_{42}$ in muscle cells were engineered – one with the temperature sensitive promoter and one that constitutively expresses $A\beta_{42}$. Originally only the constitutive model was created, but issues related to the age-dependent nature of the phenotype arose, leading to the creation of the temperature inducible strain (Lublin and Link).

The existence of two types of $A\beta_{42}$ expressing worms allows for a more complete understanding of Alzheimer's disease. Those expressing $A\beta_{42}$ pan-neuronally can be tested for changes in sensory abilities. One of the most important sensory abilities used by *C. elegans* to navigate in their environment is chemosensation. Chemosensation is used to detect the presence of food, predators, and potential mates. These environmental cues are detected by pairs of neurons located in the head of the worm called the amphid chemosensory neurons (Bargmann, Chemosensation in C. elegans). These neurons are displayed in Figure 7. Tkalčić et. al discovered that human Alzheimer's patients have deficits in odor identification abilities when compared to other elderly non-Alzheimer's patients, highlighting the translational nature of research into Alzheimer's effect on chemosensation (Tkalčić, Spasić and Ivanković).

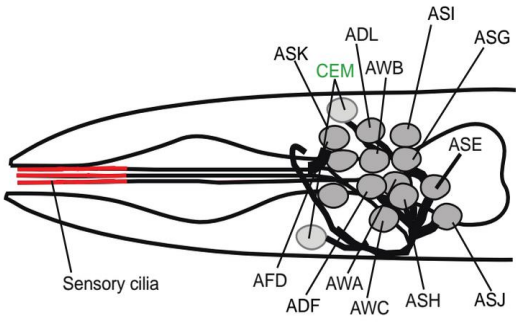

Figure 7. *C. elegans amphid sensory neurons (Hart and Chao)*

Worms expressing $A\beta_{42}$ in muscle cells, however, have been shown to have movement dysfunction, growth retardation, and shortened lifespans. These models allow for an investigation into the more physical symptoms of Alzheimer's disease, such as impaired fine motor skills (Wolozin, Gabel and Ferree). While memory loss is considered by many the hallmark symptom of Alzheimer's disease, the decreased coordination and mobility experienced by Alzheimer's patients is a significant complication that must also be addressed by researchers.

2.4 Treatment Extracts

One advantage of using *C. elegans* as a model system for discovering potential therapeutics is the fact that the worms eat *E. coli* grown in liquid media. This allows researchers to administer compounds of interest via the *E. coli* media solution. This is called the LB Medium Method of drug delivery (Zheng, Ding and Li). In our study, *C. elegans* expressing $A\beta_{42}$ were treated with various extracts through oral ingestion to determine whether an extract was able to mitigate behavioral symptoms caused by the $A\beta_{42}$ expression. One criticism of this method is that the uptake of the drug is dependent on how much food the worms eat, unlike delivery studies with larger animals where exact doses can be administered either orally or intravenously. Zheng et al. analyzed *C. elegans* uptake of a drug using various methods of administration and found that the concentration of the drug following administration was comparable to levels observed in mice studies (Zheng, Ding and Li).

Due to the prevalence of Alzheimer's disease as well as other cognitive disorders related to aging, many groups have begun to investigate the possibility of using natural extracts to prevent or reverse cognitive decline. Polyphenols are one class of extracts being examined, in part because they are cheap and readily available in modern diets (Bastianetto and Quirion). Polyphenols can be found in a variety of plants, and are known to have both antioxidant and anti-inflammatory properties. As both oxidative stress and brain inflammation have been hypothesized as possible causes of Alzheimer's disease, polyphenols have the potential to be used as therapeutics (Manach, Scalbert and Morand). Preliminary research also suggests that most polyphenols are capable of crossing the blood brain barrier and localizing within brain tissue (Vauzour). If they are found to be effective, polyphenols could represent a powerful and cost effective therapeutic tool for the prevention or treatment of Alzheimer's disease. The two polyphenols investigated in this study were punicalagin and tannic acid.

2.4.1 Punicalagin

Punicalagin is a polyphenol commonly found in pomegranates. The name punicalagin is derived from the words *Punica granatum*, the scientific name for pomegranate. While punicalagin can be found in other plants, the highest concentration is found in pomegranates. Pomegranate is known to have several health benefits because of its high concentration of antioxidants, as well as its antibacterial and antifungal properties. These properties can aid in cardiovascular protection and anti-aging. As an antioxidant, punicalagin neutralizes free radicals, therefore having a positive effect on aging by reducing aging symptoms, such as dementia. The theory of the role of free radicals in aging is that unstable oxygen atoms, having one unpaired electron in their outer shell, are in search of a complementary partner and thus are extremely chemically reactive, which destroys cells. Punicalagin attaches and binds to these free radicals, inhibiting them from damaging other cells. Other benefits of punicalagin include prevention of low density lipoprotein (LDL) cholesterol oxidation, protection against UV radiation, and reduction of blood pressure (Gailee Nutritionals).

At the University of Huddersfield in Germany, Dr. Olajide and his coworkers have established that punicalagin can help reduce neuro-inflammation in non-neuronal brain cells called microglia. This inflammation is one proposed cause of dementia and Alzheimer's disease. Rat primary microglia cells were isolated and tested with the extract at 5-40 μM for 30 minutes. Researchers found that punicalagin inhibited TRAF-6 mediated neuroinflammation, providing evidence to its possibility as a possible therapeutic for neuroinflammatory diseases such as Alzheimer's disease and Parkinson's disease (Olajide, Kumar and Velagapudi).

2.4.2 Tannic Acid

Tannic acid is another polyphenol, formed from a class of molecules called gallotannins. This polyphenol shares many properties of punicalagin, most importantly the antioxidant activity. It is already widely used in medicine, specifically for treating burns, poisoning, and diarrhea. Tannic acid is commonly found in red wine, meat products, and many plants, but is toxic at very high levels in the blood stream (Khan, Ahmad and Hadi).

Many studies have already been conducted in regards to using tannic acid to treat cancer, and it has been shown to reduce the risk of tumor formation (Khan, Ahmad and Hadi). Research has also shown that some wine-derived polyphenols can inhibit the formation of $A\beta_{42}$ *in vitro*. More specifically, tannic acid was shown to both inhibit $A\beta_{42}$ formation and destabilize $A\beta_{42}$ that

was already formed. The effective concentration (EC_{50}), or concentration that induced a response halfway between the baseline and maximum effect, was found to be below 0.1 μM (Ono, Hasegawa and Naiki). These *in vitro* tests were promising, and such low concentrations could easily be administered to patients without nearing toxic levels. Although the mechanism of action remains unclear, tannic acid is a suitable compound to study for the treatment and prevention of Alzheimer's disease.

3 Methodology

The following chapter outlines the methodology used in this project including the strains of *C. elegans* that were tested and the assays used to characterize effects on behavior.

3.1 Worm Strains

All strains used in this study were obtained from the *Caenorhabditis* Genetics Center (CGC). The wild-type strain, isolated in Bristol, UK, termed N2, was used to record the baseline data for all assays. The two strains of Alzheimer's disease *C. elegans* used in this study exhibited pan-neuronal or pan-muscular expression of a human $A\beta_{42}$ transgene. The pan-neuronal strains were tested using chemotaxis and avoidance assays, while the pan-muscular strains were used in a thrashing assay. Strain CL2355 (*smg-1*^{ts}; dvIs50 [pCL45 (*snb-1*::Aβ 1-42::3' UTR(long) + *mtl-2*::GFP]) is a heat inducible neuronal mutant due to the temperature-sensitive *smg-1*^{ts} background. It expresses $A\beta_{42}$ under the synaptobrevin (*snb-1*) promoter in all neurons when exposed to a heat shock of at least 23°C. The control for this chemosensory mutant is strain CL2122 (*smg-1*^{ts}; dvIs15 [(pPD30.38) *unc-54*(vector) + (pCL26) *mtl-2*::GFP]), which contains the same inserts and transgenes as CL2355 excluding the human $A\beta_{42}$ transgene. The pan-muscular strains were CL4176, CL2006, and CL802. CL4176 (*smg-1*^{ts}; dvIs27 [(pAF29)p*myo-3*::Aβ (1-42)::let-? 3'UTR) + (pRF4)*rol-6*(su1006)]) is a heat inducible strain due to *smg-1*^{ts} background that expresses $A\beta_{42}$ at 23°C, causing muscular deficiency. CL2006 (dvIs2 [pCL12(*unc-54*/human Aβ peptide 1-42 minigene) + pRF4]) constitutively expresses $A\beta_{42}$ without the need for a heat induction. CL802 (*smg-1*(cc546); *rol-6*(su1006)) is the control strain for CL4176, because it possesses the same *smg-1* background, without the $A\beta_{42}$. Furthermore, CL802 is also a control strain for CL2006 because it possesses the *rol-6* gene, which gives rise to the roller phenotype. This phenotype causes the worms to be helically twisted and roll when they move (CGC).

3.2 Worm Maintenance

C. elegans were grown on standard Nematode Growth Medium (NGM) plates seeded with the OP50 strain of *E. coli* grown in LB Media as the food source (Stiernagle, 2006). Worms were grown on plates with or without rescue extracts, and maintained at either 20°C (N2 strain) or 16°C (N2, CL2122, CL2355, CL4176, CL2006, and CL802 strains). Although 20°C is the most commonly accepted temperature for *C. elegans* growth, the temperature sensitive background of the strains expressing Alzheimer's phenotypes necessitated growth at 16°C.

Worms were transferred to new plates when food became scarce in order to prevent starvation. Transfer was accomplished with a homemade worm pick made with a flattened platinum wire mounted in the tip of a glass Pasteur pipette. The end of the wire was flame sterilized and then gently lowered to pick up one or more worms. The wire was then brought to the surface of a fresh, seeded NGM plate and held in place until the worm crawled onto the agar. All worm maintenance practices done are commonly used in the worm community as detailed by WormBook (Stiernagle, 2006).

3.3 Addition of Rescue Extracts to LB Media and OP50

In order to treat *C. elegans* with various extracts, each extract had to be diluted to an appropriate concentration. The extracts were delivered to the worms by mixing them with their food in LB media. The concentrations used for each extract were determined by searching primary literature.

3.3.1 Punicalagin

Based on the work by Oladije et al., 2.7 mg of 40% punicalagin was diluted in 0.27% ethanol and then added to 100 mL of ultrapure water. This solution was then filtered sterilized using a 0.20 µm SteritopTM filter unit. In order to achieve a final concentration of 2.7 mg/200 mL or 1.2 x 10^{-5} M, 100 mL of LB media previously inoculated with OP50 *E. coli* was added to the sterile punicalagin solution.

3.3.2 Ethanol Control

As a control for the punicalagin solution, a control LB media with 0.27% ethanol was made. 270 µL of 100% ethanol was added to 100 mL of media and OP50 solution.

3.3.3 Tannic Acid

Based on the work by Ono et al., 3.4 mg of tannic acid was measured and diluted in 100 mL of ultrapure water. 10 mL of this solution was added to 90 mL of ultrapure water. Finally, 10 mL of this dilution was then added to 90 mL of LB media, resulting in a final concentration of 0.034% tannic acid. In contrast to the methods used to make the punicalagin solution, the LB media added had not been previously inoculated with OP50 *E. coli*. The tannic acid and LB media solution was then filter sterilized using a 0.20 µm SteritopTM filter unit. This sterile tannic acid and LB media solution was then inoculated with OP50 *E. coli* and incubated overnight at 37°C to achieve a final concentration of 2 x 10^{-5} M.

3.4 Heat Shock Procedure

Worms were initially grown at 16°C. 20-30 L1 staged animals were passed onto a new seeded plate. There are two methods used to induce the $A\beta_{42}$ expression by temperature upshift.

The first method utilized a water bath to upshift the temperature. Two strips of parafilm were wrapped tightly around the edge of the plate to seal the gap between the plate and the lid. The plates were inverted and placed in a 23°C water bath. The plates were collected under a tube rack, and a 1 kg weight was placed on top of the rack. This kept the plates fully submerged and at a constant water pressure throughout the duration of the temperature upshift. Plates were left in the water bath for 36-48 hours. After at least 36 hours had passed, plates were taken out of the water bath and the parafilm was removed. Plates were wiped dry and left inverted on the bench top at room temperature for one hour. After one hour, worms were tested with the avoidance assay.

The second method used to induce the $A\beta_{42}$ expression was by use of a 25°C incubator. Following passage of L1 staged worms to a new seeded plate, plates were inverted and placed in a 25°C incubator for 36-48 hours. After at least 36 hours, plates were taken out of the incubator and were let to acclimate to room temperature for one hour, after which, the worms were tested on either the chemotaxis or thrashing assays. The water bath heat shock method was tested for the chemotaxis methods, however baseline results were lower than normal and the plates had a tendency to become contaminated, so the incubator was used instead. These problems were not seen with the avoidance assay, so the water bath continued to be used.

3.5 Avoidance Assay

10 adult worms were picked from a seeded plate onto an unseeded NGM plate. After one minute of acclimation time, the worms were tested with the drop assay as originally described by Hilliard in 2004 (Hilliard, Bergamasco and Arbucci).

In this assay, 10 µl glass capillaries were pulled over a flame to create a thin micro needle tip. The capillary was then inserted into a micro-pipetting apparatus. A 5 nanoliter drop was delivered by mouth to the tail of a forward moving worm. Capillary action caused the drop to surround the worm, traveling forward from the tail to the head of the worm, eventually reaching the chemosensory neurons located at the anterior tip of the animal. A response of a full reversal, omega turn, or turn greater than 90° within four seconds of drop administration was scored as an avoidance response. If the worm continued forward motion after drop administration, this was

scored as “no avoidance” (Hart and Chao). A depiction of the responses of no avoidance and avoidance is displayed in Figure 8.

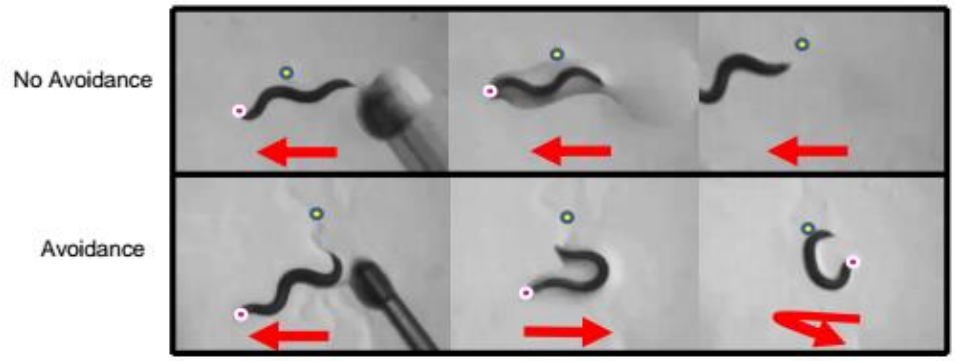

Figure 8. *Example of avoidance and no avoidance in the drop assay. The red arrow points towards the direction of forward motion. (Adapted from Chute).*

M9 buffer containing 0.1% sodium dodecyl sulfate (SDS) was used as the repellant, with M9 buffer used as a vehicle control. SDS is a synthetic organic chemical often used in cleaning products whereas M9 buffer is a salt solution often used to wash and prepare worms for freezing (JoVE Science Education Database). To make 0.1% SDS, 5 µl of 20% SDS was added to 995 µl of M9 buffer. M9 buffer is made up of 3.0g KH_2PO_4, 6.0g Na_2HPO_4, 0.5g NaCl, and 1.0g NH_4Cl mixed with enough water to bring the mixture to 1L (Riddle, Blumenthal and Meyer).

One drop of the SDS was placed on each worm, and the response was recorded. A timer was set and the worms were left alone for two minutes. After two minutes, one drop of SDS was again placed on each worm and responses were recorded. This was done three times with each solution, resulting in six drops of solution being placed on each worm tested.

In order to determine the ratio of worms that avoided the avoidance chemical to those that did not avoid, an avoidance index was calculated. The index is normalized to exclude worms that also avoided the control chemical. As depicted in Figure 9, this is accomplished by calculating the number of worms that avoided the control subtracted from the number of worms that avoided the SDS. This was then divided by the total number of worms tested to produce an avoidance index between 0 and 1. A higher avoidance index indicated a higher level of avoidance to that chemical.

$$Avoidance\ Index = \frac{Worms\ Avoiding\ SDS - Worms\ Avoiding\ M9}{Total\ Worms\ Tested}$$

Figure 9. *Equation to calculate avoidance index.*

3.6 Chemotaxis Assay

At the beginning of this study, an attraction assay was used to determine the length of time that the worms spent directly within an attractant solution as compared to the time spent in a vehicle control of ultrapure water. The initial intention was to assess the effect of the neuronal expression by CL2355 through extrapolating attraction data as an indication of chemotaxis ability. Unfortunately, this extrapolation could not provide the necessary data because it did not highlight each worm's chemosensory abilities, so a different assay had to be utilized (See Appendix 6-3 for procedure of attraction assay). An assay designed to directly measure chemotaxis in *C. elegans* was selected and used for the remainder of the study (Ward).

The final chemotaxis assay used was based on an assay developed by Samuel Ward in 1973. After testing both diacetyl and isoamyl alcohol (IAA) to determine which chemical had a more robust response in the chemotaxis assay, it was decided that IAA (10^{-2} dilution) would be the chemical used going forward. The protocols for dilutions of both chemicals can be found in Appendix A and B.

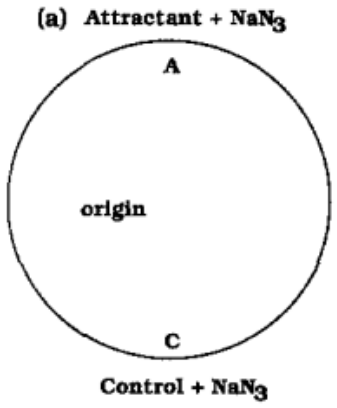

Figure 10. *Design of chemotaxis assay. Worms are placed on the origin spot (Bargmann, 1993).*

Assay plates were made from 2% agar, 5 mM KPO_4 pH 6.0, 1 mM $CaCl_2$, and 1 mM $MgSO_4$, poured into a 10 cm petri dish. Two small marks were made 180 degrees apart on the bottom of the plate. In Figure 10, these marks are noted as A and C. 1 µL of 1 M sodium azide (NaN_3) was placed on each mark in order to paralyze worms once they were in close proximity to the marks.

To prepare for the assay, approximately 100 worms were removed from a seeded plate and washed three times using S. Basal, a mild solution used to remove traces of food or eggs from the worms, in a 1.5 mL microcentrifuge tube. S. basal is a salt buffer made of 5.85g NaCl, 1g K_2HPO_4, 6g KH_2PO_4, 1mL cholesterol in ethanol, and enough water to fill to 1L (Stiernagle). Following the three washes, the worms were washed once with ultrapure water to remove any residual S. Basal. 10 µL of the washed worms were then transferred to the origin point (located in the center of the plate) as seen in Figure 10. Excess liquid was then carefully removed using a KimWipe. 1 µL of IAA and ultrapure water was placed on the A and C marks, respectively. The lid of the plate was immediately closed following the placement of the

chemicals to contain the odor of the chemicals, as IAA is volatile. The assay was run for one hour, during which, the plates were continually checked for clumping of the worms at the origin point. If clumping was observed the worms were gently dispersed with a pick. At the end of the hour, the number of paralyzed worms in each chemical was counted. A chemotaxis index was then calculated using the following equation:

$$Chemotaxis\ Index = \frac{Worms\ at\ Attractant - Worms\ at\ Control}{Total\ Worms\ Tested}$$

Figure 11. *Equation to calculate the chemotaxis index of one test plate.*

3.7 Thrashing Assay

A thrashing assay developed by Brenner et al. in 1974 was optimized to test impaired movement caused by the expression of pan-muscular $A\beta_{42}$. An adult worm was picked from a seeded NGM plate and placed on an unseeded NGM plate, where it was allowed to crawl around for one minute in order to remove any residual OP50 *E. coli*. It was important for the bacteria to be eliminated, as it could alter the worm's movement during the assay.

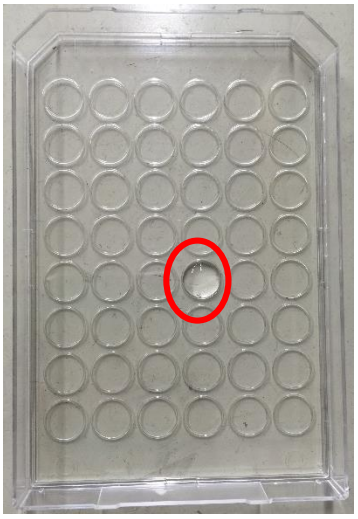

Figure 12. *Lid of a 48-well plate used for thrashing assay with 100 µl of M9 buffer.*

After a few assay design iterations, the lid of a 48-well plate was chosen for the setup, with the circular outlines of the lid filled with 100 µl of M9 buffer, as seen in Figure 12.

When the isolated worm was clear of *E. coli*, it was picked from the unseeded plate and placed in the middle of an M9 drop. The worm was left in the drop for one minute to allow it to acclimate to the environment. The thrashes of the worm were then recorded for one minute using the WormLab Image Acquisition software by MBF Biosciences. Following one minute of thrashing, the video was analyzed using the WormLab Analysis software. The worm's mid-point bending angle, shown in green in Figure 13, was analyzed by the software. A total of four worms were tested for each strain and condition.

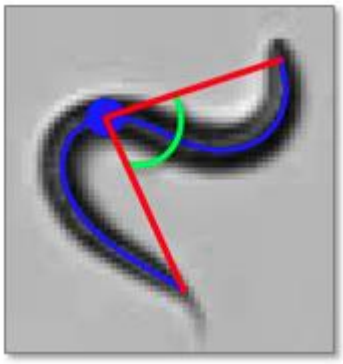

Figure 13. *Display of mid-point bending angle of C. elegans (WormLab).*

4 Results and Discussion

Based on the previously described methodology, the following chapter outlines the results obtained from the avoidance, chemotaxis, and thrashing assays of worms grown on OP50, OP50 and Punicalagin, OP50 and ethanol, and OP50 and Tannic Acid, as well as comparisons between treatment conditions.

4.1 Baseline Testing

In order to determine baseline data the three assays were run with wild type (N2), control (CL2122, CL802), and transgenic strains (CL2355, CL4176). These strains were grown on NGM plates seeded with LB media + OP50 *E. coli*. Testing was performed on unseeded plates.

4.1.1 Avoidance Assay

Testing the chemosensory ability of N2 worms using the avoidance assay yielded an average avoidance index of 0.76 ± 0.02973, when propagated at 20°C, consistent with the results from Hillard et al. in 2002 (Hilliard, Bergamasco and Arbucci). Figure 14 shows the avoidance indices for all worms grown on normal OP50. The avoidance index of N2 worms grown at temperatures other than 20°C was slightly lower, however this lower avoidance index was consistent between N2 and CL2122. CL2355 at 16°C was consistent with the N2 at 20°C.

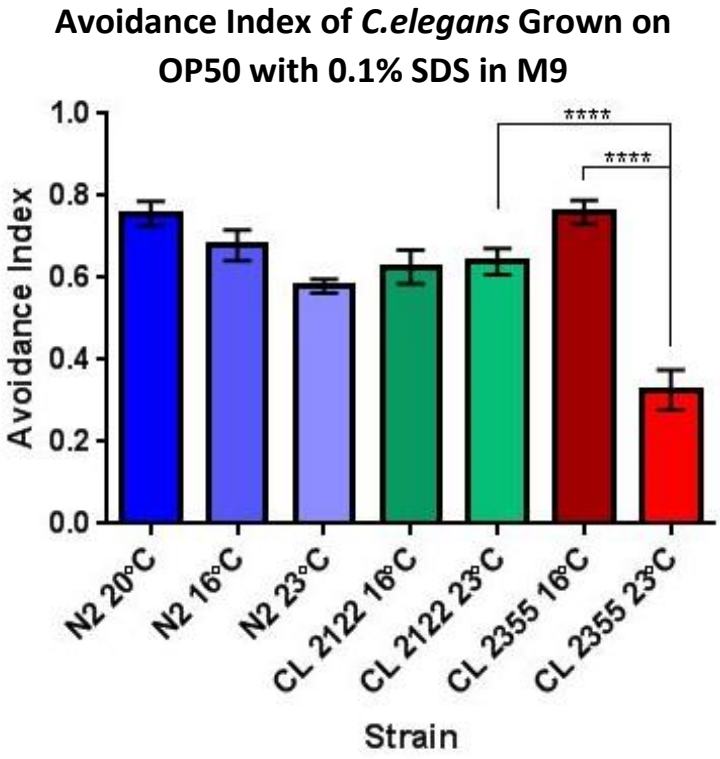

Figure 14. *Avoidance index of N2, CL2122, and CL2355 at various temperatures on NGM plates.*

Evidence of chemosensory deficit caused by the expression of $A\beta_{42}$ peptide is demonstrated by the significantly lower avoidance index of CL 2355 worms at 23°C (0.33 ± 0.04877), compared to CL 2122 at 23 °C (0.64 ± 0.03239, as well as compared to CL2355 without a temperature upshift, 0.76 ± 0.02860 ($p < 0.0001$, one-way ANOVA, Bonferroni's correction). This indicates that $A\beta_{42}$ expression caused the worms to lose their ability to sense and respond to the aversive cue, indicating a chemosensory deficit.

4.1.2 Chemotaxis Assay

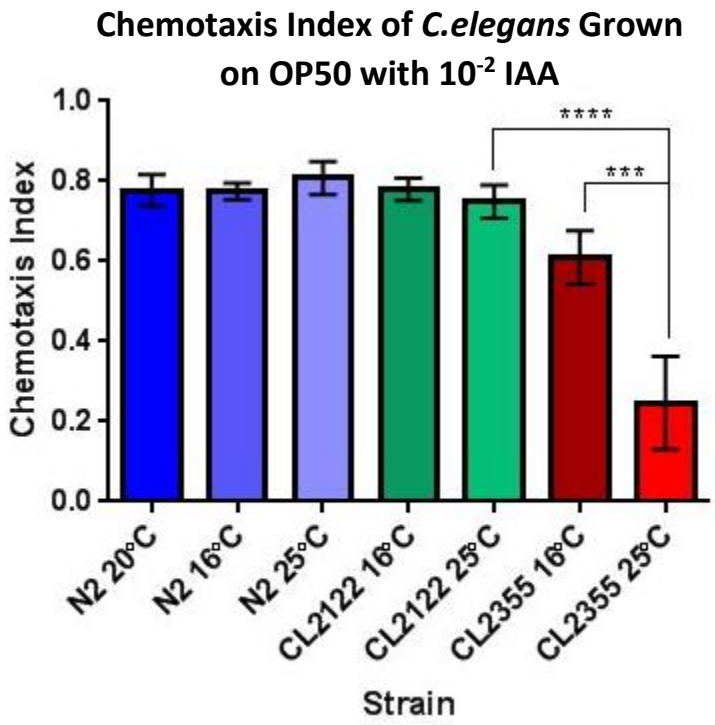

Figure 15. *Chemotaxis Index of N2, CL 2122, and CL 2355 at various temperatures on regular NGM plates.*

N2 worms propagated at 20°C are expected to have a chemotaxis index of about 0.80 (Bargmann, Hartwieg and Horvitz, Odorant-selective genes and neurons mediate olfaction in C. elegans). In this study, a chemotaxis of 0.78 ± 0.03952 was obtained for N2 worms grown at 20°C, consistent with these results. Figure 15 shows the chemotaxis indices for all worms grown on normal OP50.

The chemotaxis index of N2 worms grown at 20°C, 16°C (0.78 ± 0.02048) and 25°C (0.81 ± 0.04081) was not significantly different. CL2122 worms had a similar chemotaxis index at each temperature (0.78 ± 0.02835 at 16°C and 0.75 ± 0.04133 at 25°C), while CL2355 was slightly lower with the upshift of temperature (0.25 ± 0.1162). The expression of $A\beta_{42}$ peptide following temperature upshift led to a reduced chemotaxis index compared to CL2355 without the upshift (0.61 ± 0.06706) ($p = 0.0003$, one-way ANOVA, Bonferroni's correction). There is a significant difference of the chemotaxis index between CL 2122 at 25 °C and CL 2355 at 25°C ($p < 0.0001$, one-way ANOVA, Bonferroni's correction). This again indicated a reduction in chemosensory abilities in response to $A\beta_{42}$ expression.

4.1.3 Thrashing Assay

A thrashing assay was performed to evaluate the effect of Aβ expression on muscle cells. Figure 16 shows the average thrash count per minute for N2, CL802, and CL4176 grown on OP50. The average thrash count per minute of an N2 worm grown at 20°C was 139.50 ± 19.53 thrashes. CL 802 had a significantly reduced thrash count per minute at 52.50 ± 6.035 thrashes. This can be

explained by the roller phenotype that both CL 802 and CL 4176 have. This phenotype causes the worms to roll in order to move, instead of crawl.

The effect of the $A\beta_{42}$ peptide can be seen when CL 4176 was propagated at 25°C. There is a significant difference in the number of thrashes with and without the upshift in temperature (53.25 ± 11.46 at 16°C and 20.50 ± 7.005 at 25°C) ($p = 0.0417$, one-way ANOVA, Bonferroni's correction). This indicates that the expression of $A\beta_{42}$ caused impaired movement. There was no significant difference between CL 802 grown at different temperatures, nor between CL802 and CL4176 following temperature upshift.

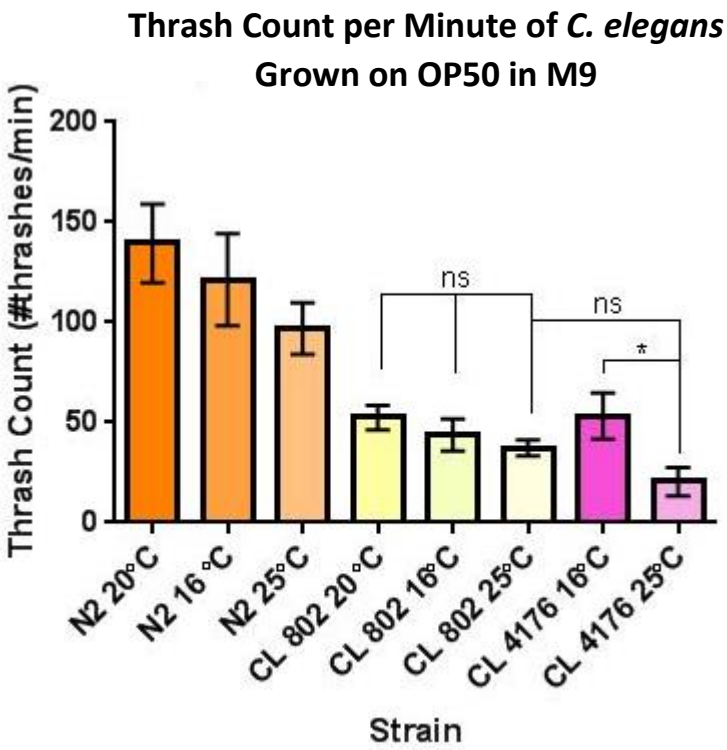

Figure 16. *Thrash count per minute of N2, CL802, and CL4176 at various temperatures on regular NGM plates.*

4.2 Punicalagin

To assess the effects of punicalagin on the phenotypes seen in strains expressing $A\beta_{42}$ peptide, all strains were grown on NGM plates seeded with OP50 and 12 µM punicalagin with 0.27% ethanol. The ethanol was added to dissolve the punicalagin in the OP50. To control for the added ethanol, all strains were grown on OP50 with 0.27% ethanol and tested with each assay.

4.2.1 Avoidance Assay

The same avoidance assay was used to test the chemosensation ability of worms treated with punicalagin. N2 worms grown on OP50 with punicalagin at 20°C had an avoidance index of 0.49 ± 0.1076. This result was lower than expected because of one trial with an abnormally high control response. However, the rest of the data was consistent with normal values of about 0.80. Figure 17 shows the avoidance indices for all worms grown on OP50 with punicalagin.

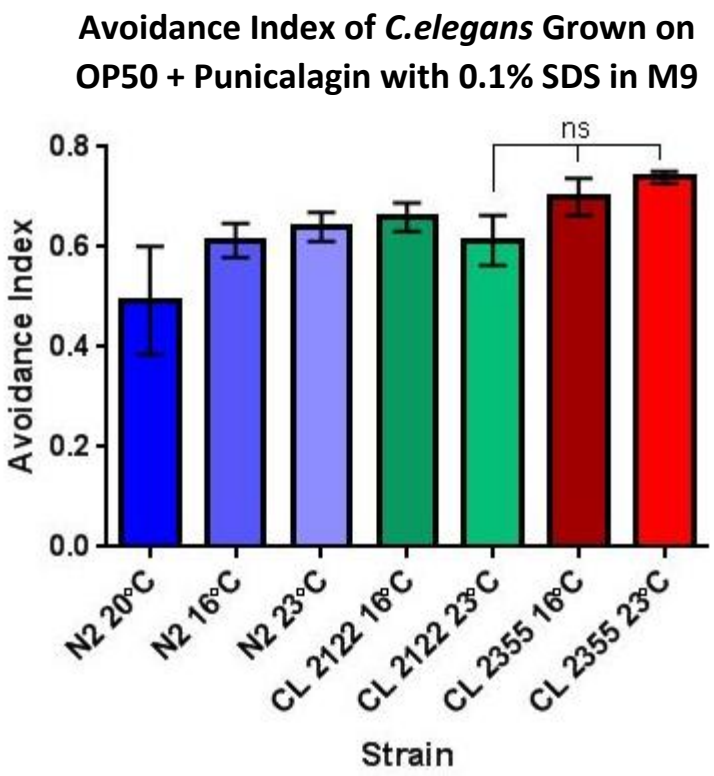

Figure 18. *Avoidance index of N2, CL2122, and CL2355 at various temperatures on NGM plates with punicalagin.*

The lower avoidance index caused by the pan-neuronal expression of $A\beta_{42}$ was alleviated when worms were grown on OP50 with punicalagin, as evidenced by the fact that the avoidance index of CL 2355 at 23°C, 0.74 ± 0.01235, was not significantly different from that of CL 2355 at 16°C, 0.70 ± 0.03651 or of CL 2122 at 23°C, 0.61 ± 0.05004 ($p > 0.9999$ and $p = 0.1845$, one-way ANOVA, Bonferroni's correction). This indicates that the $A\beta_{42}$ expressing worms grown on OP50 + punicalagin were able to sense and respond to the aversive cue at levels similar to worms not expressing $A\beta_{42}$. The avoidance indices for worms grown on OP50 with 0.27% ethanol to control for the punicalagin tests are shown in Figure 18.

There was a significant difference in the avoidance index of CL 2355 at 23°C (0.19 ± 0.03237) to CL 2355 at 16°C (0.73 ± 0.01215) ($p < 0.0001$, one-way ANOVA, Bonferroni's correction), indicating a deficit in the worms' chemosensation, similar to the results gathered during baseline testing. The

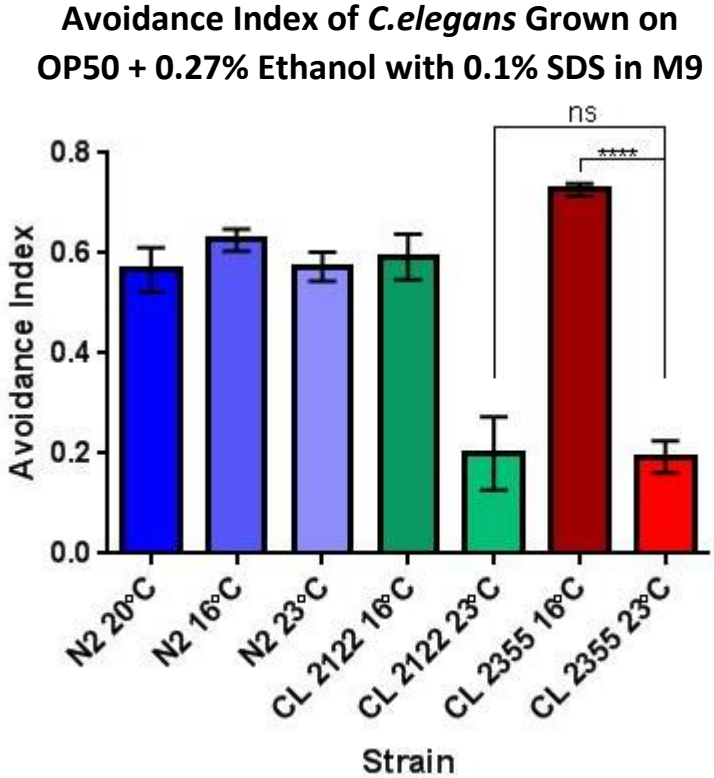

Figure 17. *Avoidance index of N2, CL2122, and CL2355 at various temperatures on NGM plates with 0.27% ethanol.*

CL 2122 worms grown at 23°C also exhibited a lower avoidance index (0.20 ± 0.07296), which was a result of high levels of response to the control chemical.

4.2.2 Chemotaxis Assay

Similar to the results of baseline testing, N2 strains grown on OP50 at 20°C with punicalagin had a chemotaxis index of 0.75 ± 0.04636. Figure 19 shows the chemotaxis indices for all worms grown on OP50 with punicalagin.

Similar to the results seen from the avoidance assay, punicalagin showed a rescuing effect. There was no significant difference between the chemotaxis indices of CL 2355 at 25°C (0.56 ± 0.02169) and at 16°C (0.58 ± 0.04353) ($p > 0.9999$, one-way ANOVA, Bonferroni's correction). This indicated that when grown on OP50 with punicalagin, the chemosensory deficit previously seen during baseline testing was eliminated. All strains were also grown on OP50 with 0.27% ethanol to serve as control. Figure 20 shows the chemotaxis indices for all worms grown on OP50 with 0.27% ethanol.

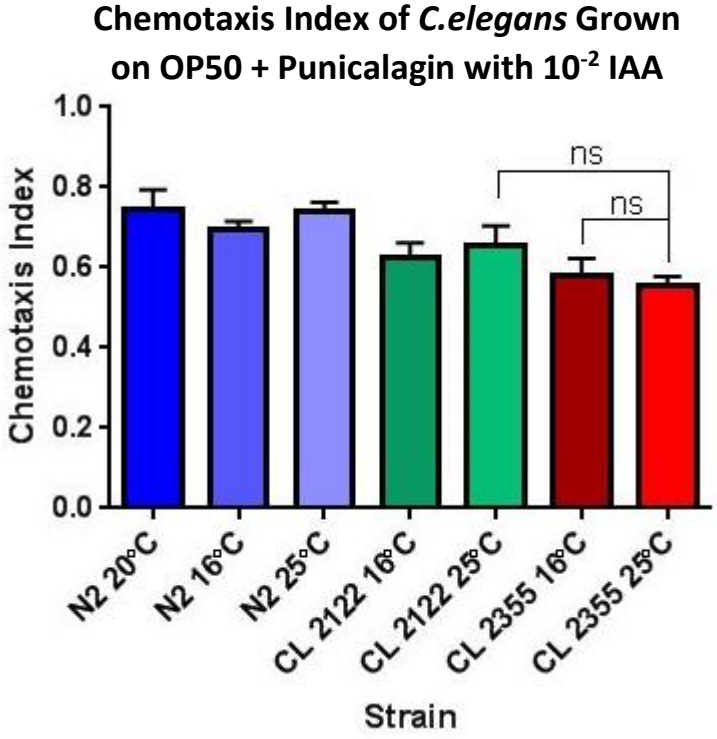

Figure 19. *Chemotaxis Index of N2, CL 2122, and CL 2355 at various temperatures on NGM plates with punicalagin.*

The results obtained for worms grown on OP50 with 0.27% ethanol were similar to those of baseline. There was a significant difference in the chemotaxis indices of CL 2355 at 25°C (0.26 ± 0.01526) and CL 2355 at 16°C (0.55 ± 0.03098) ($p < 0.0001$, one-way ANOVA, Bonferroni's correction), which indicates a deficit in the worms' chemosensation. This ethanol control result indicated that the rescuing effects seen in Figure 19 were only due to the addition of punicalagin, and not due to the presence of the ethanol.

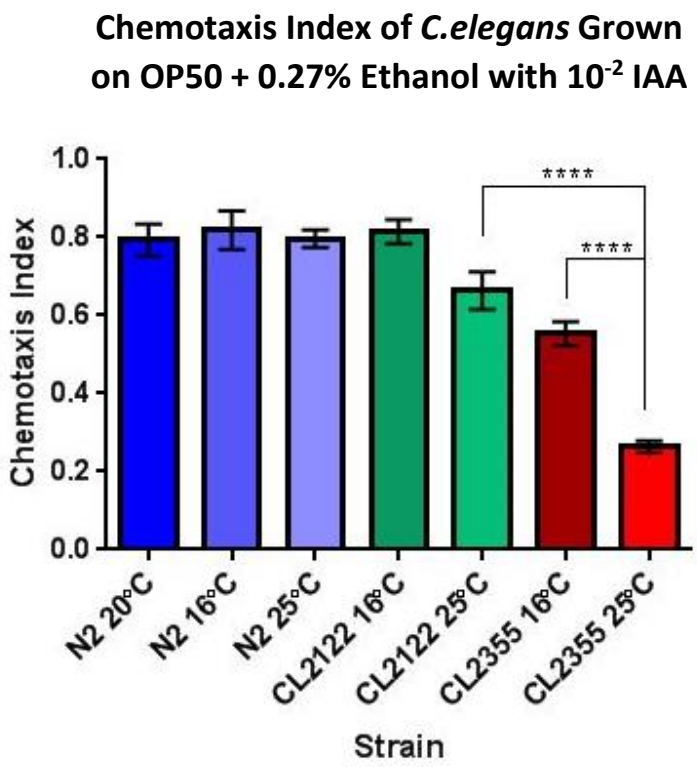

Figure 21. *Chemotaxis Index of N2, CL 2122, and CL 2355 at various temperatures on NGM plates with 0.27% ethanol.*

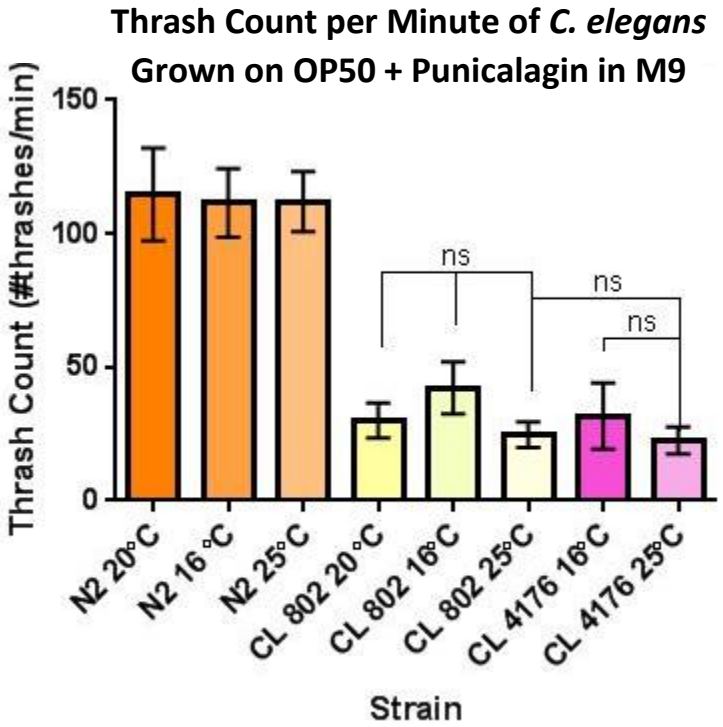

Figure 20. *Thrash count per minute of N2, CL802, and CL4176 at various temperatures on NGM with punicalagin plates.*

4.2.3 Thrashing Assay

This assay was performed to evaluate the effect of punicalagin on the worms expressing $A\beta_{42}$ pan-muscularly. Figure 21 shows the thrash count per minute of all worms grown on OP50 with punicalagin.

In contrast to the results from the avoidance and chemotaxis assays, the addition of punicalagin did not have an effect on the

impaired movement seen in worms expressing $A\beta_{42}$ peptide in their muscle cells. There was no significant difference in the thrash count per minute of CL 4176 at 25°C (22.50 ± 5.041) to CL 4176 at 16°C (31.75 ± 12.32) and to CL 802 at 25°C (24.75 ± 4.820) ($p = 0.5497$ and $p > 0.9999$, one-way ANOVA, Bonferroni's correction). This indicated that punicalagin had no rescuing effect in the worms expressing $A\beta_{42}$ pan-muscularly. Figure 22 shows the thrash count per minute for all worms grown on OP50 with 0.27% ethanol.

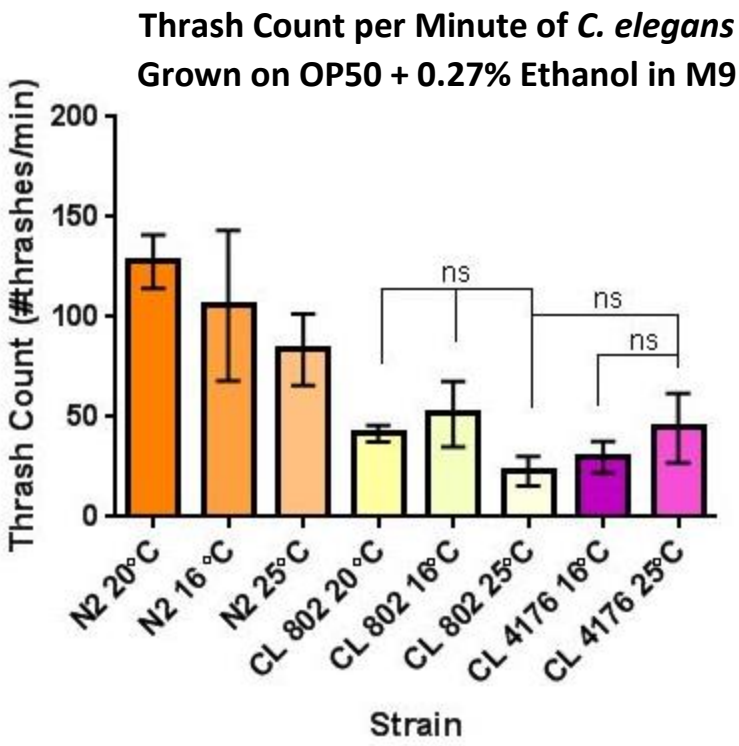

Figure 22. *Thrash count per minute of N2, CL802, and CL4176 at various temperatures on NGM plates with 0.27% ethanol.*

The results obtained from the thrashing assay revealed similar thrash counts to those obtained in baseline testing. There was no significant difference between CL 4176 at 25°C (44.5 ± 17.39) to CL 4176 at 16°C (30.00 ± 7.927) and CL 802 at 25°C (23.00 ± 7.439) ($p = 0.5764$ and $p > 0.9999$, one-way ANOVA, Bonferroni's correction), still indicating impaired movement of the worms expressing $A\beta_{42}$.

4.3 Tannic Acid

To assess the effects of tannic acid on the phenotypes seen in strains expressing $A\beta_{42}$ peptide, all strains were grown on NGM plates seeded with OP50 and 20 µM tannic acid.

4.3.1 Avoidance Assay

The avoidance assay was used to test the effects of tannic acid on the worms' chemosensation. N2 worms grown on OP50 with tannic acid yielded an avoidance assay of 0.69 ± 0.02868. Figure 23 shows the avoidance indices for all worms grown on OP50 with tannic acid.

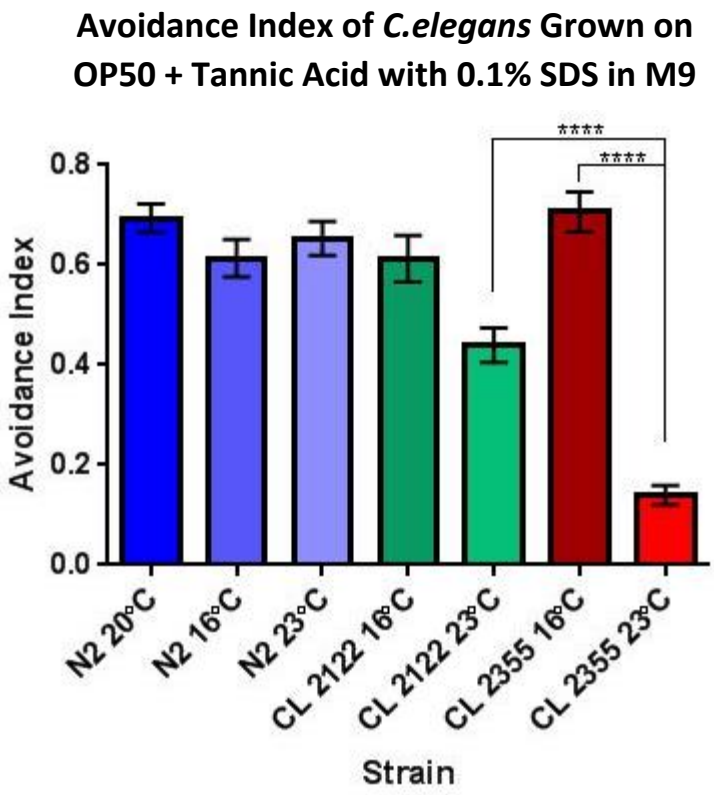

Figure 23. *Avoidance index of N2, CL2122, and CL2355 at various temperatures on NGM plates with tannic acid.*

Following an upshift in temperature, CL 2355 grown on OP50 with tannic acid showed impaired chemosensation as seen with the avoidance index similar to those of baseline testing (0.14 ± 0.01947). Furthermore, there was a significant difference in the avoidance indices of CL 2355 at 23°C to CL 2355 at 16°C (0.71 ± 0.04002) and to CL 2122 at 23°C (0.44 ± 0.03403) ($p < 0.0001$ for both, one-way ANOVA, Bonferroni's correction). This indicated that tannic acid has no rescuing effect on the neuronal deficits caused by $A\beta_{42}$ expression.

4.3.2 Chemotaxis Assay

The chemotaxis assay was also used to evaluate the effects of tannic acid on the worms' chemosensation abilities. N2 worms grown on OP50 at 20°C with tannic acid had a chemotaxis index of 0.70 ± 0.02545. Figure 24 shows the chemotaxis indices for all worms grown on OP50 with tannic acid.

Similar to results obtained during baseline testing, the chemotaxis index of CL 2355 at 25°C (0.26 ± 0.03493) was significantly lower than CL 2355 at 16°C (0.73 ± 0.08363) and to CL 2122 at 25°C (0.71 ± 0.04931) ($p < 0.0001$ for both, one-way ANOVA, Bonferroni's correction). This indicted that tannic acid had no effect on the deficit seen in the chemosensation ability of worms expressing $A\beta_{42}$.

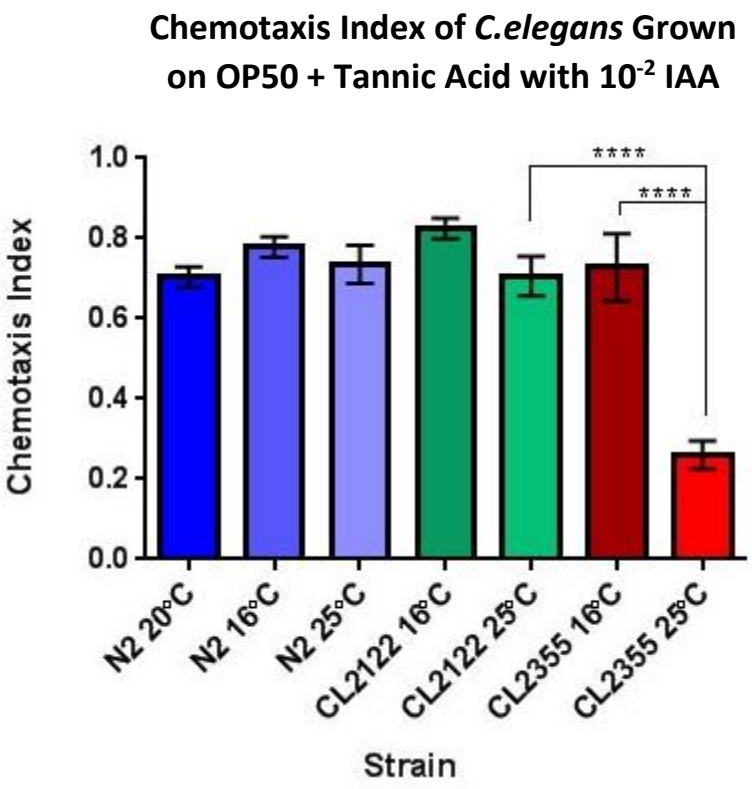

Figure 24. *Chemotaxis Index of N2, CL 2122, and CL 2355 at various temperatures on NGM plates with tannic acid.*

4.3.3 Thrashing Assay

This assay was performed to evaluate the effect of tannic acid on the muscular abilities of the worms. Figure 25 shows the thrash count per minute of all worms grown on OP50 with tannic acid.

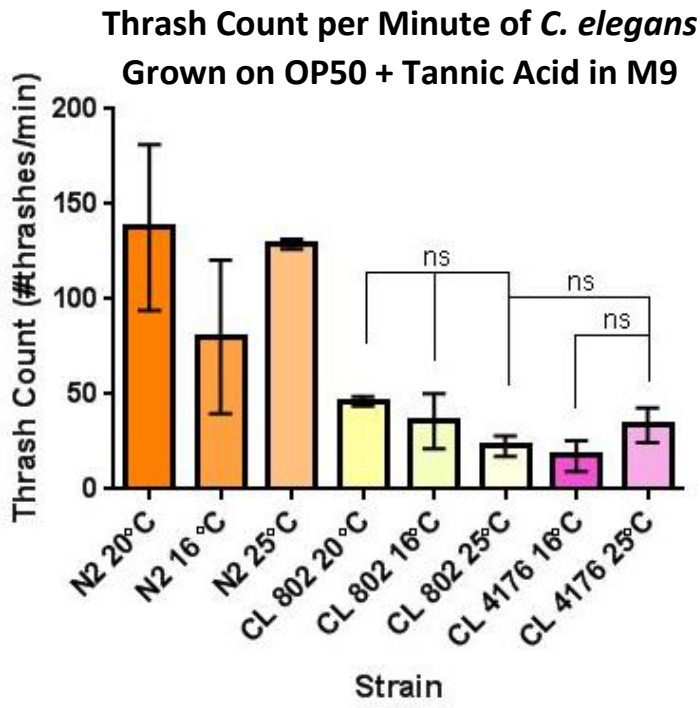

Figure 25. *Thrash count per minute of N2, CL802, and CL4176 at various temperatures on regular NGM plates with tannic acid.*

The addition of tannic acid yielded no effect on the impaired movement of worms expressing $A\beta_{42}$. This was seen from the not significantly different thrash count per minute of CL 4176 at 25°C (33.75 ± 9.196) to CL 4176 at 16°C (17.50 ± 8.067) and CL 802 at 25°C (22.75 ± 5.406) ($p = 0.6091$ and $p > 0.9999$, one-way ANOVA, Bonferroni's correction).

4.4 Treatment Comparisons

Based on the results, punicalagin demonstrated a neuroprotective effect against the behavioral deficit seen in worms expressing $A\beta_{42}$ pan-neuronally. However, it does not show the same protective effect in worms expressing $A\beta_{42}$ pan-muscularly. In contrast, tannic acid did not show any rescuing effect on worms expressing $A\beta_{42}$ either pan-neuronally or pan-muscularly. Figure 26 depicts the comparison between avoidance indices of CL 2122 and CL 2355 grown at all conditions.

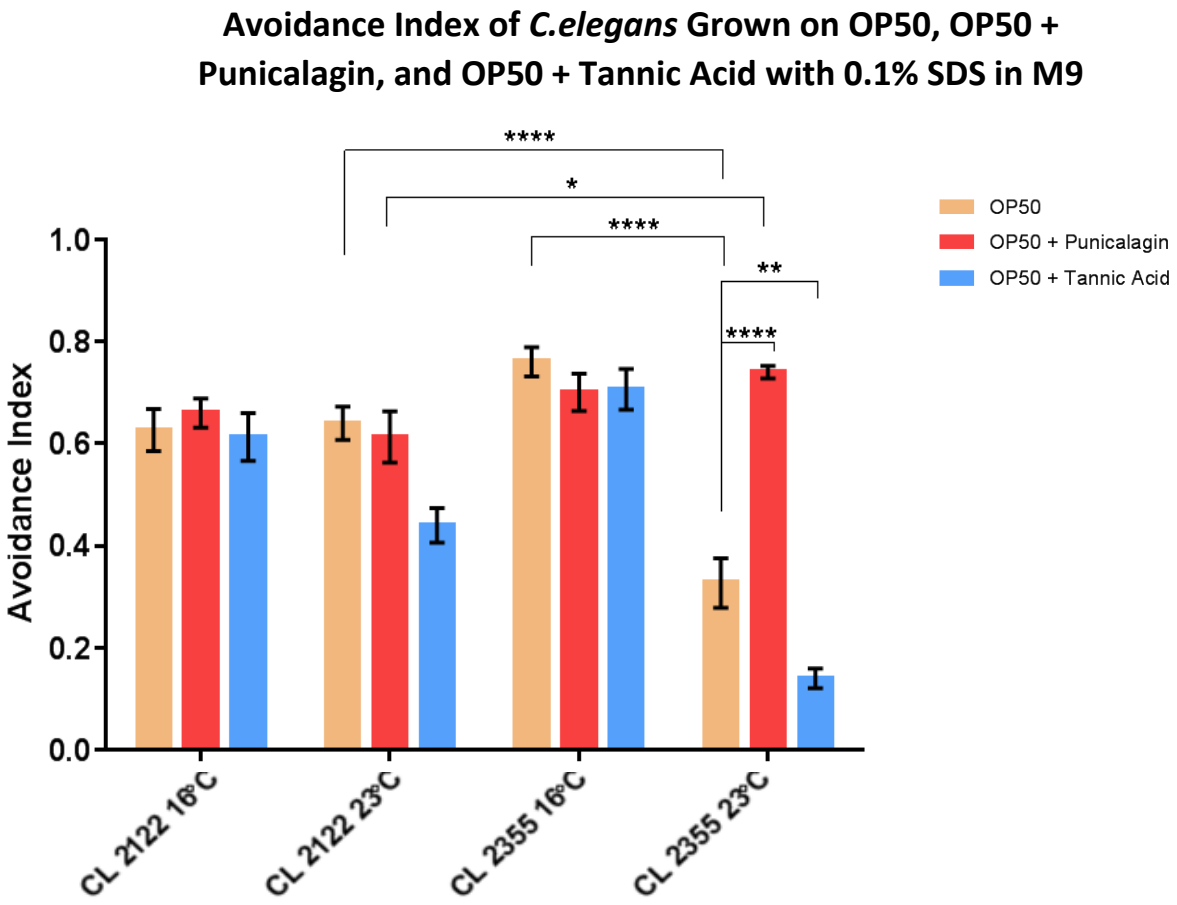

Figure 26. *Comparison of avoidance indices of CL2122 and CL2355 grown at various temperatures on NGM plates with OP50, OP50 + Punicalagin, and OP50 + Tannic Acid.*

Evidence of the effect of $A\beta_{42}$ expression is seen with the significant difference of the avoidance indices of CL 2355 on OP50 with and without an upshift in temperature. $A\beta_{42}$ expression reduces the avoidance index from 0.76 ± 0.029 to 0.33 ± 0.049 ($p < 0.0001$, one-way ANOVA, Bonferroni's correction). This indicates a defect in the worms' chemosensation since the worms were unable to sense the repellent chemical as aversive. However, when CL 2355 was grown on OP50 with punicalagin at 23°C, the behavioral defect was alleviated, evidenced by the avoidance index returning to 0.74 ± 0.013, a high level of avoidance. In contrast, CL 2355 grown on OP50 with tannic acid with the upshift in temperature did not display the same effect. These worms had a significantly lower avoidance index (0.14 ± 0.019) compared to CL 2355 grown on OP50 at 23°C (0.33 ± 0.049) ($p = 0.0021$, two-way ANOVA, Tukey's correction). This suggests that tannic acid may have had a toxic effect on the worms' behavior or development, which could affect their ability to sense and respond to the aversive cues.

The second assay used to test the worms' chemosensory abilities was the chemotaxis assay. Figure 27 depicts the comparison between chemotaxis indices of CL 2122 and CL 2355 grown at all conditions.

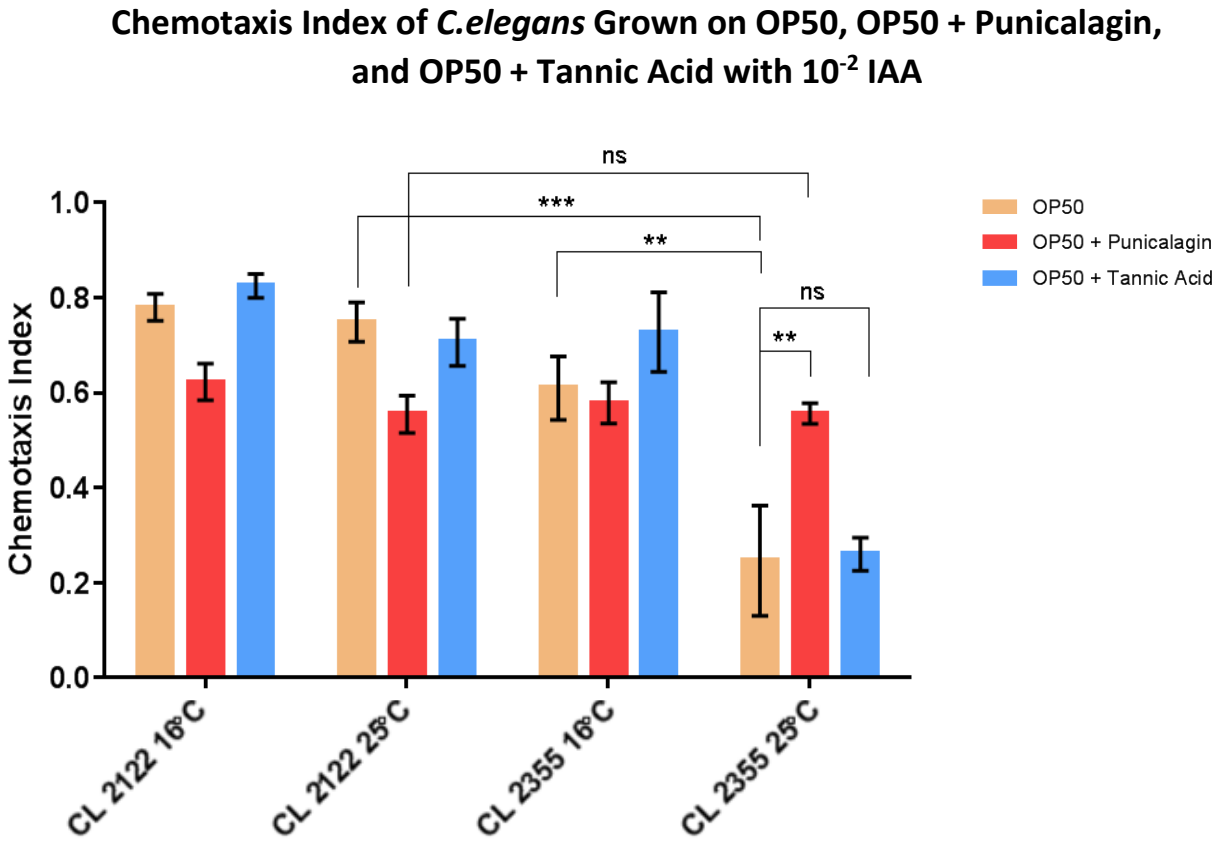

Figure 27. *Comparison of chemotaxis indices of CL2122 and CL2355 grown at various temperatures on NGM plates with OP50, OP50 + Punicalagin, and OP50 + Tannic Acid.*

The effect of $A\beta_{42}$ expression is seen with the significant difference of the chemotaxis indices of CL 2355 on OP50 with and without an upshift in temperature, which reduces the chemotaxis index from 0.61 ± 0.067 to 0.25 ± 0.116 ($p = 0.0041$, one-way ANOVA, Bonferroni's correction). Consistent with the results gathered from the avoidance assay, this indicates a defect in the worms chemosensory as the worms were not attracted to the attractant chemical. Similarly, punicalagin alleviated the behavioral defect, as seen by a higher chemotaxis index of 0.56 ± 0.022, which is significantly different from the chemotaxis index of CL 2355 grown on OP50 alone with the upshift in temperature ($p = 0.0012$, two-way ANOVA, Tukey's correction). Furthermore, CL 2355 grown on OP50 with tannic acid with the upshift in temperature did not display any effect (0.26 ± 0.035), seen from its low chemotaxis index that is not significantly different from that of

CL 2355 on OP50 with the upshift in temperature (0.25 ± 0.116) ($p = 0.9842$, two-way ANOVA, Tukey's correction).

The last assay used in this project was the thrashing assay, which tested the effect of $A\beta_{42}$ on the worms' muscular abilities. Figure 28 depicts the thrash count per minute of CL 4176 under all tested conditions.

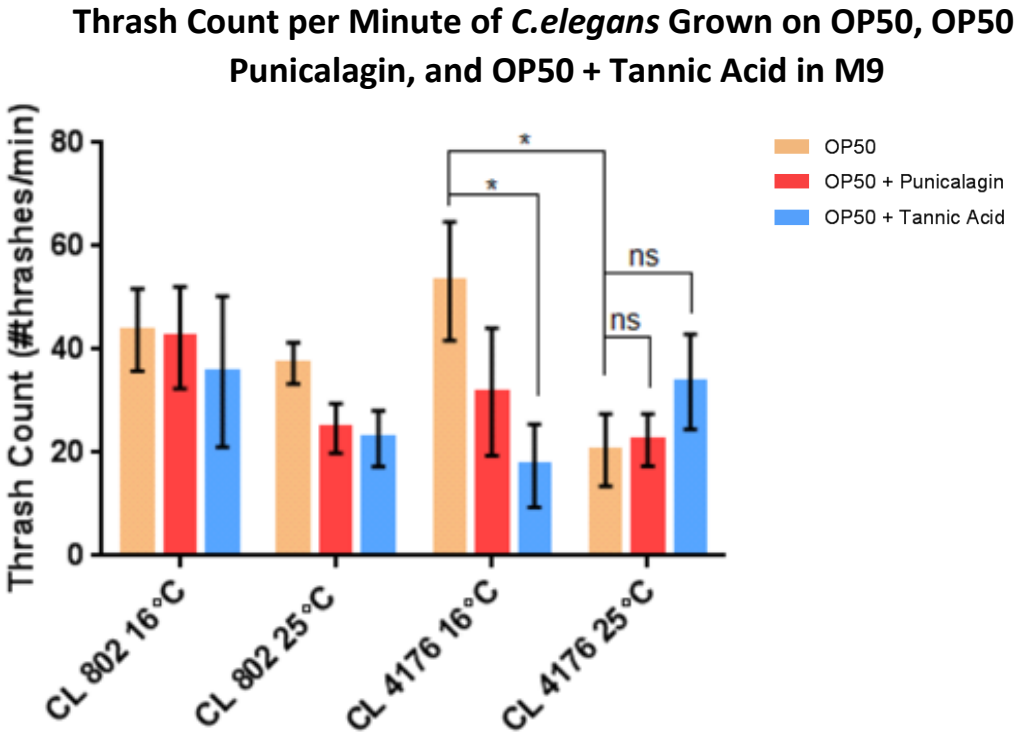

***Figure 28**. Comparison of thrash count per minute of CL 802 and CL4176 grown at various temperatures on NGM plates with OP50, OP50 + Punicalagin, and OP50 + Tannic Acid.*

The effect of $A\beta_{42}$ expression on muscle cells can be seen from the significantly different thrash count per minute of CL 4176 grown on OP50 with and without an upshift in temperature. The thrash count per minute went from 53.25 ± 11.456 thrashes per minute to 20.50 ± 7.005 thrashes per minute ($p = 0.0349$, one-way ANOVA, Bonferroni's correction). This behavioral defect caused by the $A\beta_{42}$ expression was characterized as impaired movement. Furthermore, neither extract tested had any effect on the behavioral defect, as seen from the similar thrash count per minute from worms grown on OP50 to OP50 with punicalagin or tannic acid with the upshift in temperature ($p > 0.9999$ and $p = 0.4793$, two-way ANOVA, Bonferroni's correction). Therefore, in contrast to the alleviation of behavioral defects seen in worms expressing $A\beta_{42}$ pan-neuronally, none of the extracts tested displayed the same effect on worms expressing $A\beta_{42}$ pan-muscularly.

There was also a significant difference in the thrash count per minute between CL 4176 grown on OP50 and OP50 with tannic acid without an upshift in temperature nor expression of $A\beta_{42}$ ($p = 0.0159$, two-way ANOVA, Bonferroni's correction). This suggests that tannic acid might have a toxic effect on the worms' movement response even without $A\beta_{42}$ expression.

It should be noted that the method used to analyze the results of the thrashing assay in this study has not been standardized. The method of analysis used was developed by the researchers of this project and therefore further improvements can be made to produce a more robust representation of the data.

5 Conclusions and Future Work

From this study we have verified three behavioral assays that can be used to evaluate the behavioral effects of $A\beta_{42}$ expression in *C. elegans*. Two of these assays test the chemosensation of worms expressing $A\beta_{42}$ in neuronal cells, while the third tests the movement of worms expressing the protein in muscle cells. Versions of these tests have been used before for behavioral testing, but not in the context of an Alzheimer's disease model. Each assay showed a reduction in neuroperformance when the animals were heat-shocked to initiate $A\beta_{42}$ expression. This indicates that these assays are appropriate tools to measure these behavioral phenotypes. These phenotypes are analogous to human Alzheimer's disease symptoms of impaired olfactory ability and dysfunction of movement.

From the results, we are able to conclude that the polyphenol punicalagin mitigates the behavioral defects in *C. elegans* caused by $A\beta_{42}$ protein expression in neuronal cells. This therapeutic effect was not seen in worms expressing $A\beta_{42}$ protein in muscle cells. There are various possible explanations for this dissimilar effect. One explanation is that the worms expressing $A\beta_{42}$ in muscle cells exhibited a roller phenotype caused by expression of the gene *rol-6*. This gene itself impairs movement of the worms with or without $A\beta_{42}$ expression (Kramer, French and Park), so it would likely be more difficult for any potential rescue effect to be observed. The assay used to analyze the impaired movement of these worms could also be improved upon. Data analysis was done using thrash count, but WormLab software has a variety of options for analyzing worm movement, including speed, direction of movement, worm position, and track length. It is possible that one of these other analysis methods would more accurately display differences between the test strains and treatment groups. A third possible explanation is that the mechanism of action is different in neuronal cells than muscle cells. Studies have shown that $A\beta_{42}$ is more likely to accumulate in plaques in the brain, while $A\beta_{40}$, which is more soluble, tends to accumulate between muscle cells (Serrano-Pozo, Frosch and Masliah). Punicalagin may have been able to interfere with $A\beta_{42}$ expression of the neuronal cells but not the muscle cells and so only rescued the behavior in strains expressing $A\beta_{42}$ pan-neuronally.

Tannic acid, unlike punicalagin, was unable to mitigate any of the behavioral effects. This was surprising, as previous studies have implicated tannic acid as a good candidate for Alzheimer's disease therapeutics (Ono, Hasegawa and Naiki). Other studies have shown that tannic acid can be toxic at high levels (Khan, Ahmad and Hadi), providing some insight into these unexpected results.

In the avoidance assay, the worms responded to the control chemical at relatively high levels, similar to the worms grown on OP50 and ethanol. Qualitative observation of these worms also indicated that not all worms placed on a plate survived to adulthood, and that the worms grew more slowly. If there was a developmental defect caused by the tannic acid this may have been a confounding variable that further worsened the worms' chemosensory abilities. Further understanding of this effect can be achieved by testing various concentrations of tannic acid to determine if lower concentrations continue to cause this effect. We tested concentration of tannic acid was chosen based on previous studies performed with neuronal cells (Ono, Hasegawa and Naiki). We chose to increase the concentration used on these cells, because we believed that *C. elegans* would be able to withstand a higher dose, as it is a fully developed organism. It is possible that the concentration chosen was too high, so a return to the concentration of 0.1μM used by Ono in 2004 could give more insight to this. Researchers could also use even lower concentrations, in case there were toxic effects on the cells used by Ono that could not be observed *in vitro* (Ono, Hasegawa and Naiki).

Worms were placed on OP50 with or without extracts as eggs and allowed to hatch and mature all while feeding on the extract being tested. This leads us to believe that the punicalagin prevented the symptoms from ever manifesting in the worms, rather than cured symptoms that were already present. Since most cases of Alzheimer's disease are diagnosed after the disease has taken hold of the patient, it would be useful to test punicalagin as a retroactive cure as well, where the $A\beta_{42}$ expression is induced prior to treatment with punicalagin. In addition to investigating the ability of punicalagin to alleviate behavioral symptoms retroactively, this could also give some insight into the mechanism behind the therapeutic effect of the extract. If punicalagin were simply blocking $A\beta_{42}$ gene expression then retroactive treatment might not be successful. If punicalagin is removing the $A\beta_{42}$ post-accumulation or reducing oxidative stress, the retroactive treatment might still have a similar effect. Further analysis to understand why punicalagin had this neuroprotective effect would be necessary before any clinical applications of the compound could be investigated. This could include a biochemical analysis of how much punicalagin is ingested by the worms and how it is distributed and digested.

Researchers could take this project a step further and investigate the role of gender bias in the symptoms shown from the $A\beta_{42}$ peptides. In humans, women are approximately twice as likely to develop Alzheimer's disease as men (Alzheimer's Association). Since *C. elegans* is a sexually

dimorphic organism, researchers could investigate whether there are sex-specific behavioral defects caused by the expression of $A\beta_{42}$ peptides. While not a male-female system, the differences between male and hermaphrodite *C. elegans* could provide some insight into the different mechanisms driving $A\beta_{42}$ expression and accumulation in different genders.

In order to fully validate the use of CL 2355 and CL 4176 as models of human Alzheimer's disease, researchers should investigate whether or not the $A\beta_{42}$ that is expressed following growth at a temperature upshift is actually forming the amyloid plaques that are a hallmark of the disease. Lublin and Link explain that it is unlikely that *C. elegans* models can completely mimic human Alzheimer's disease pathology (Lublin and Link), however the accumulation into plaques is an important aspect of the disease. This also underscores the need for further testing of punicalagin and any other extracts implicated in invertebrate models in mammalian models before looking into the effects on human patients.

This study provides a preliminary analysis of the effects of punicalagin and tannic acid on *C. elegans* expressing $A\beta_{42}$ in either neuronal or muscular cells. This is a promising platform for furthering our understanding of this devastating disease. *C. elegans* provides a system that can show altered behavioral phenotypes caused by $A\beta_{42}$ expression and can be studied at the molecular level to understand the mechanisms and pathways driving these changes. It is also valuable that these worms show deficits in multiple aspects of behavior, allowing for validation of results between assays and for the study of various effects of the disease. This study successfully characterized the behavioral effects of $A\beta_{42}$ expression in three separate assays and found that punicalagin has the potential to mitigate the effects of this expression. Hopefully future research will continue to make breakthroughs in the understanding of Alzheimer's disease and the search for a cure.

6 Appendix

6.1 Appendix A: Isoamyl Alcohol Dilution

In order to create 1 µM solution of isoamyl alcohol (3-methyl-1-butanol), the desired mass to be diluted was first calculated from its molecular weight. Isoamyl alcohol (IAA)'s molar mass of 88.15 g/mol was divided by 4 x 10^4 moles. The mass to be diluted to achieve a concentration of 1mM IAA with a volume of 25 mL was 0.00220375 g or 22.0375 x 10^{-4} g. This value was confirmed using Sigma-Aldrich's Mass Molarity Calculator. The mass of 22.0375 x 10^{-4} g was then divided by its density, 0.809 g/mL (Sigma-Aldrich), to obtain the desired volume needed. Therefore, 2.724 µL of 1 M IAA was diluted with 25 mL of ultrapure water to obtain a final concentration of 1mM IAA. The solution was made in a 50 mL conical tube. Another dilution was then performed to obtain a final concentration of 1µM IAA. 9.99 µL of the 1 mM stock solution of IAA was diluted in 10 mL of ultrapure water, obtaining a final concentration of 1 µM IAA. From the 1 µM solution, aliquots of 1 mL were distributed in amber vials. The conical tubes and the amber vials were wrapped with aluminum foil to protect the stock solutions from the light and were stored at 4°C. The initial stock solution bottle of IAA was kept in the flammables container. All dilution work was performed in a chemical hood with proper ventilation to avoid inhalation of IAA.

These aliquots were used for the attraction assay during the experimentation process of the project. However, when they were used for chemotaxis assay, these aliquots did not elicit the most robust index. Therefore, new concentrations of IAA were made for the chemotaxis assay. The working stock had a final concentration of 10^{-2}. This was done by adding 100 µl of the concentrated stock IAA (98%) into 900 µl of ultrapure water for the first dilution, creating a concentration of 10^{-1}. Then, 100 µl of the first dilution of IAA (10^{-1}) were added to another 900 µl of purified water to achieve the second dilution of 10^{-2} concentration. These concentrations were decided to be the best as it produces the most robust chemotaxis response. The working stock of IAA was placed in an amber vial, which was stored in the 4°C fridge. All work was performed in the hood to avoid inhalation of IAA.

6.2 Appendix B: Diacetyl Dilution

In order to create 1 µM solution of diacetyl (butane-2,3-dione), the desired mass to be diluted was first calculated from its molecular weight. Diacetyl's molar mass of 86.09 g/mol was divided by 4 x 10^4 moles. The mass to be diluted to achieve a concentration of 1mM diacetyl with a volume of 25 mL was 0.00215225 g or 21.5225 x 10^{-4} g. This value was confirmed using Sigma-Aldrich's Mass Molarity Calculator. The mass of 21.5225 x 10^{-4} g was then divided by its density, 0.981 g/mL (Sigma-Aldrich), to obtain the desired volume needed. Therefore, 2.190 µL of 1 M diacetyl was diluted with 25 mL of ultrapure water to obtain a final concentration of 1mM diacetyl. The solution was made in a 50 mL conical tube. Another dilution was then performed to obtain a final concentration of 1µM diacetyl. 9.99 µL of the 1 mM stock solution of diacetyl was diluted in 10 mL of ultrapure water, obtaining a final concentration of 1 µM diacetyl. From the 1 µM solution, aliquots of 1 mL were distributed in amber vials. The conical tubes and the amber vials were wrapped with aluminum foil to protect the stock solutions from the light and were stored at 4°C. The initial stock solution bottle of diacetyl was kept in the flammables container. All dilution work was performed in a chemical hood with proper ventilation to avoid inhalation of diacetyl.

6.3 Appendix C: Attraction Assay

The assay was developed by Srinivasan et al in 2008 and uses a template as seen in Figure 29. 24 hours prior to the assay, NGM plates were seeded with LB media containing OP50. Aseptic technique was used to seed plates to avoid contamination. Three to four drops of media were added to the center of each plate using sterile Pasteur pipettes. A bent glass pipette was flame sterilized and used to spread the media. This was accomplished by holding the pipette in place and spinning the plate continuously until the media was evenly spread over the agar. Plates were stored at room temperature for 20 minutes, and then inverted and stored overnight in a 20°C incubator. Two circles with a diameter of 5 mm are drawn on the template to mark the placement of the vehicle control and attractant chemicals. The X spots located near the circles are where the worms were initially placed in the beginning of each trial. 5 worms are placed on each X spots.

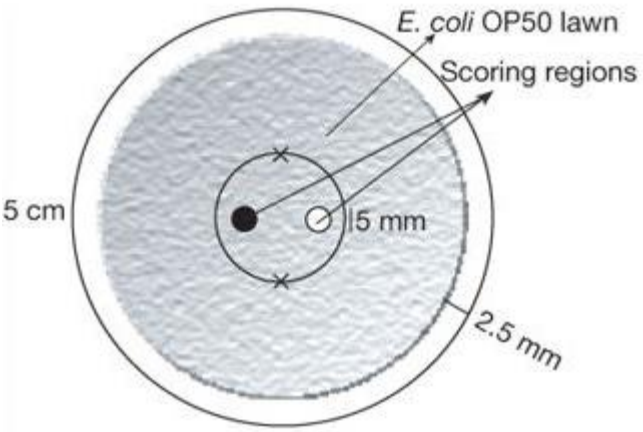

Figure 29. *Attraction assay template on NGM testing plate (Based on Srinivasan, 2008).*

0.6 µl of the attractant chemical, either isoamyl alcohol or diacetyl, was added to one circle of the template on the seeded lawn plate. Isoamyl alcohol is a colorless alcohol that attracts wild-type worms and diacetyl is an organic compound with a buttery odor that illicit the same attraction in wild-type worms. 0.6 µl of ultrapure water was added to the other circle on the plate to act as the vehicle control. Once all of the chemicals had been added, worms were added to the test plate. The lid of the plate was removed and a modified plastic bowl was placed around the plate to avoid in any effects from any cross-breeze in the lab during recording as seen in Figure 30. Each plate was recorded for 20 minutes with a total of 5 plates tested for one session. The video was then analyzed by scoring the amount of time of each visit to the control versus attractant drops.

Figure 30. *Microscope setup to record the attraction assay.*

6.4 Appendix D: Supplemental Data of CL 2006 on All Conditions

In addition to CL 4176, the strain CL 2006 was also used as a constitutive Aβ strain that expresses Aβ pan-muscularly. This strain was tested using the thrashing assay to evaluate the effect of Aβ on the worms' movement and also to assess the effects of punicalagin and tannic acid on the worms' muscle cells.

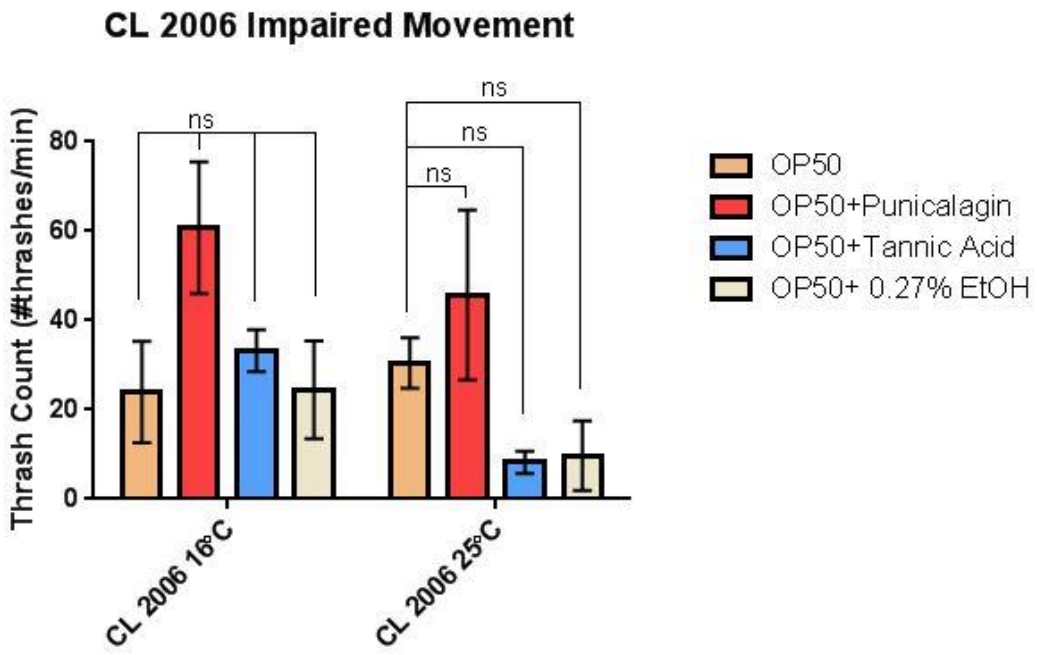

Figure 31. *Thrash count per minute of CL 2006 grown on with either OP50, OP50 + Punicalagin, OP50 + Tannic Acid, or OP50 + 0.27% ethanol at various temperatures.*

As seen in Figure 31, which outlines the thrash count per minute of CL 2006 at all conditions on various temperatures, the impaired movement caused by the $A\beta_{42}$ can be seen from CL 2006 at 25°C on OP50, which shows a thrash count of 30.5 ± 5.679 thrashes per minute. Furthermore, there are no significant difference between the thrash count per minute of CL 2006 at 16°C grown on OP50 with those grown on OP50 with punicalagin, OP50 with tannic acid, or OP50 with 0.27% ethanol ($p = 0.0679$, $p > 0.9999$, and $p > 0.9999$, two-way ANOVA, Bonferroni's correction). Therefore, it is evident that none of the treatments had an effect on the impaired movement caused by the $A\beta_{42}$ peptide.

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